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Proteases secreted by *Gnathostoma binucleatum* degrade fibronectin and antibodies from mammals

N. VIBANCO-PÉREZ*, M. DE J. DURÁN-AVELAR, J. F. ZAMBRANO-ZARAGOZA, G. H. VENTURA-RAMÓN

Universidad Autónoma de Nayarit, Unidad Académica de Ciencias Químico Biológicas y Farmacéuticas, Cd. de la Cultura, Amado Nervo s/n, Edificio CEMIC 05, CP 63190, Tepic, Nayarit, México, *E-mail: *novipe@hotmail.com*

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Summary

Gnathostomiasis is a prevalent zoonosis in humans in some regions of the world. The genus *Gnathostoma* is considered an accidental parasite for humans; *G. binucleatum* is the endemic species in Nayarit, Mexico. This work was designed to determine the proteolytic activity of the excretory-secretory products (ESP) of advanced third-stage larvae (ADVL3) of *Gnathostoma binucleatum* against human fibronectin and antibodies from human and sheep. Our findings showed protease activity against human fibronectin as well as sheep and human gamma globulins of the ESP at molecular weights of 80 and 56 kDa. The proteases found in the ESP of *G. binucleatum* are thus candidate molecules for consideration as pathogenic elements, owing to the fact that they destroy proteins of the host tissue, which probably allows them to migrate through those tissues and degrade molecules involved in the humoral immune response.
Keywords: *Gnathostoma binucleatum*; proteases; mammal proteins; excretory-secretory products

Introduction

Human gnathostomiasis is produced by several species of nematodes of the genus *Gnathostoma* that, as adults, live in the esophagus or stomach of diverse mammal species, both wild and domestic, where they form open tumors. The advanced third-stage larvae (ADVL3) are infective to humans who become accidental hosts. In such cases, the parasites do not reach the adult stage and thus behave as migration larvae (Lamothe-Argumedo & Osorio-Sarabia, 1998).

The interaction mechanisms between the ADVL3 of *G. binucleatum* and the host have not yet been determined clearly; hence, little is known of the pathogenesis of this nematode. However, Miyazaki (Miyazaki, 1960) has described that the damage caused by parasites of the genus *Gnathostoma* may be due to a combination of factors, including the mechanical effects of migration by the parasite, the elimination of toxic substances, proteolytic enzymes, and the host's inflammatory reaction.

It has been reported that when the larvae are ingested by a host, they lose their cystic envelope in the stomach, perforate the stomach wall, and then migrate to the liver. Once there, they can disperse to any part of the body (Lamothe-Argumedo & Osorio-Sarabia, 1998); however, the mechanisms involved in this process remain unclear. Karanu *et al*. (1993) have proposed that nematodes migrate through tissues, whereas Dalton and Heffernan (1989) reported that both mechanical and enzymatic processes are used to penetrate the host's body.

In recent decades, the number of studies of the proteases of infectious agents has increased, due to the role of these enzymes in the biological processes involved in the pathogenesis of infectious diseases, including some parasitic types. These enzymes perform different functions, such as facilitating tissue invasion, digesting proteins, and evading the immune response (McKerrow & Doenhoff, 1988; McKerrow, 1989; Trap & Boireau, 2000; Rebeloo *et al.*, 2012). Excretory-secretory (ES) products are known to contain a variety of enzymes, such as acetylcholinesterase, superoxide dismutase, and proteases (Todorova *et al.*, 1995), and different proteases have been identified in helminthes, such as *Schistosoma mansoni* (Trap & Boireau, 2000), *Fasciola hepatica* (Dalton & Heffernan, 1989; Trap & Boireau, 2000; Smith *et al.*, 1993;Burasain *et al.*, 1997), and *Haemonchus contortus* (Karanu *et al*., 1993; Rhoads & Fetterer, 1995), *Angiostrongylus cantoniensis* (Wei *et al.*, 2011). With respect to *G. binucleatum*, proteolytic activity has been described only on gelatin (Caballero-Garcia *et al.*, 2005). Because proteases are crucial molecules for parasites, they have been proposed as potential targets of the immune response and chemotherapeutic agents, and as antigens for serodiagnosis (McKerrow & Doenhoff, 1988; McKerrow, 1989; Caballero-Garcia

et al., 2005; Campista-León *et al.*, *2012*; Uparanufraw *et al.*, 2001). The ADVL3 of *Gnathostoma* sp. are characterized by their ability to migrate in host tissues, and this must be the result of the action of the proteases that they excrete or secrete. The objective of this work, therefore, was to characterize the proteolytic activity of the ESP of the ADVL3 of *Gnathostoma binucleatum* against human fibronectin, and human and sheep gamma globulins.

Materials and Methods

A total of 742 live ADVL3 were obtained from fish known locally as *chihuil* (*Cathorops fuerthii*) acquired from fish-vendors in the city of Tepic, Nayarit, Mexico. The ground fish meat was placed in plastic bags, compressed with a metallic press, and examined against light under a stereoscopic microscope (Motic®, Japan). The ADVL3 were seen to form cysts, which were then removed manually from their cystic envelope using histological needles. They were washed several times with sterile PBS until elimination of all residues foreign to the larvae was confirmed by microscopic observation.

To confirm the identity of the parasites isolated, we followed the procedure described by Zambrano-Zaragoza *et al.* (2012). Briefly, after obtaining the ESP, genomic DNA was purified from the ADVL3 and used as a template to perform PCR with primers specific for the species: for ITS-1, Lim1657 5'-CTGCCTTTGTAC ACACCG-3' and ITS-RIXO 5-TGGCTGCGTTCTTCATCG-3'; and for ITS-2, NEWS2 5'-TGTG TCGATGAAGAACGCAG-3' and ITS2-RIXO 5'-TTCTATGCTTTAAATTCA GGGG-3'. The fragments obtained were sequenced, and these sequences were aligned with those reported for *G. binucleatum*.

To obtain the ESP, viable ADVL3 (confirmed by their movement under microscopic observation) were placed in a sterile plastic tube and 10 µL of sterile PBS were added for each larva. This mixture was incubated at room temperature (RT) for 48 h, and the supernatants were recovered and pooled. Protein concentration was determined by absorption at 280 nm and 260 nm, using an UV/Visible spectrophotometer (Beckman Coulter™, USA). The samples were then stored in aliquots at -20 °C until use (Rhoads & Fetterer, 1995; Bollang *et al.*, 1996).

To characterize the proteolytic activity of the ESP, 10 % polyacrylamide gels were copolymerized with 0.05 % bovine gelatin (Merck, Germany), 0.025 % human plasmatic fibronectin (Gibco BRL, USA), 0.025 % sheep gamma globulins (Amersham-Biosciences, USA), or 0.025 % human gamma globulins (Rockland, USA), as described by Dalton and Heffernan (1989). Afterwards, 2 µg of ESP were mixed with the sample buffer in non-denaturing conditions (1 M Tris, 50 % glycerol, 1 % bromophenol blue, pH 6.8) and resolved in copolymerized gels at 100 V for 5 h at 4 °C. Next, the gels were treated as described previously (Dalton & Heffernan, 1989; Todorova *et al.*, 1995; Gamble & Mansfield, 1996) with some modifications. Briefly, they were washed with 2.5 % Triton X-100 for 30 min at RT, and were then incubated in 100 mM Tris at pH 8.0 for 16 h, and stained with 0.04 % Coomassie Brilliant Blue (Piacenza *et al.*, 1997). Finally, the gels were washed with a hot 10 % acetic acid solution until the bands became visible. For activity against gelatin, the gels were also incubated in 0.1 M sodium citrate at pH 4.5, and activity was determined at both room temperature and 37 °C. Bands with proteolytic activity were identified using molecular weight markers as references (Bio-Rad, Laboratories, USA).

Results and Discussion

The PCR products obtained with the pairs of primers ITS-1 and ITS-2 were 900 and 600 bp, respectively. The alignment of the 421 bp product of ITS-2 showed 100 % identity with the sequences reported for *G. binucleatum* (Almeyda-Artigas *et al.*, 2000; León-Regagnon *et al.*, 2002) and 99.76% with the nucleotide sequence of a parasite of the same species obtained by biopsy (Almeyda-Artigas *et al.*, 2000), indicating that the larvae used to obtain the ESP were indeed *G. binucleatum.*

The ESP showed proteolytic activity against bovine gelatin at both pH 8 and pH 4.5. Three main degradation bands were observed, whether the incubation temperature was 37 °C or RT. Taking the molecular weight markers as reference, these bands were located at 80, 56, and 50 kDa, respectively (Fig. 1).

For the genus *Gnathostoma*, only a few proteases have been

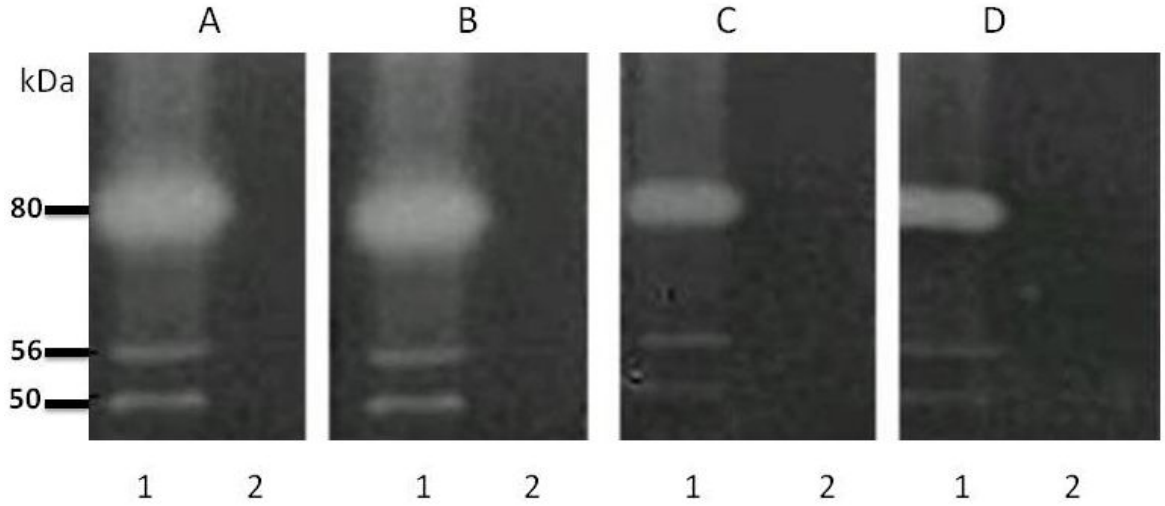

Fig. 1. Proteolytic activity of ES products of ADVL3 of *G. binucleatum* cultured for 48 h in PBS. Polyacrylamide gels copolymerized with bovine gelatin. (A) 100 mM Tris, pH 8.0 at room temperature, (B) 100 mM Tris, pH 8.0, at 37°C, (C) 0.1 M sodium citrate, pH 4.5, at room temperature, (D) 0.1M sodium citrate, pH 4.5 at 37°C. Lanes 1, proteolytic activity of 48-h incubated ES products. Lanes 2, controls (PBS).

characterized, but they include the 24 kDa, cathepsin L-like cysteine protease from *Gnathostoma spinigerum* (Kongkerd *et al*., 2008), which has been proposed for use as a specific antigen in diagnostics. In the case of *G. binucleatum*, Caballero *et al*. (2005) reported two bands with proteolytic activity against gelatin at 205 and 80 kDa. They also found that the 80 kDa protease activity was inhibited by EDTA, which allowed them to affirm that it was a metalloprotease. In line with those results, we also found that the 80 kDa band has activity against gelatin, and was inhibited by EDTA (data not shown). In contrast, the present work did not identify the 205 kDa band, but we report two more activities at 65 and 50 kDa.

The differences in the proteolytic activities observed could be due to the conditions used to obtain the ESP. Caballero-Garcia *et al*. (2005) cultured the ADVL3 in RPMI-1640 at 37 °C in a CO_2 atmosphere with a week of incubation. Our experiments used PBS and only 48 hours of incubation at RT. It has been reported that the culture media, stage of differentiation, and host are all important in the induction of proteases in nematodes (Rebello *et al*., 2012), so it the 50 and 56 kDa bands were induced in our conditions but not in those used by Caballero-Garcia *et al*. (2005), even though the culture conditions they utilized were more suitable for the survival and production of the EPS of these larva since they contained more nutrients and the presence of CO_2.

On the other hand, the ESP showed proteolytic activity at RT against human fibronectin, sheep and human gamma globulins. The 80 and 56 kDa bands showed proteolytic activity against all the substrates used, but the 50 kDa band showed activity only against gelatin (Fig. 2, lanes 1A, 1B, 1C and 1D).

The finding that the proteases of this parasite can degrade bovine gelatin at acid and base pH suggests that it could be used to migrate across distinct environments in different hosts (Díaz-Camacho *et al*., 2000); for example, from the environment of the luminal side of the stomach epithelium across the gastric wall, which has a pH gradient (Tortora & Derrickson, 2011).

Likewise, the fact that proteolytic activity was observed at a slightly alkaline pH indicates that these proteases could be used by the parasite to cross such host tissues as skeletal muscle tissue, as suggested by the clinical manifestations caused by the migration of the parasite (Martínez *et al*., 1989; Ollague *et al*., 1984; Tudor & Blair, 1971).

The bands with protease activity at 80 and 56 kDa are able to hydrolyze proteins from the extracellular matrix, suggesting that they could be involved in penetrating or migrating through tissues, as has been described for other helminthes (Berasain *et al*., 1997; Knox, 2011). The infective larvae of the nematode *Ancylostoma caninum* secrete proteases involved in migration through host tissues (Williamson *et al*., 2011); moreover, proteolytic activity by a protease against collagen and fibrinogen has been reported in the ESP of third-stage larvae of the nematode *Lagochilascaris minor* (Barbosa *et al*., 2006).

In addition, the bands with protease activity at 80 and 56 kDa were able to degrade both sheep and human gamma globulins. The presence of two proteases –cathepsin-like L1 and L2– has been demonstrated in the ESP of *Fasciola hepatica* adults that cleave human IgG type immunoglobulins close to the papain cleaving site. This has been interpreted as a protection for the parasite against the host's immunological system (Smith *et al*., 1993). Also, in *Haemonchus contortus*, the presence of multiple cysteine proteases with hydrolytic activity on IgG supports their role in immune evasion (Rhoads & Fetterer, 1995). Together, these data suggest that the proteases of *G. binucleatum* described herein could be involved in evading the immune response.

The 80 and 56 kDa bands showed proteolytic activity against more than one substrate, so each band may contain more than one protease or, alternatively, a single multifunctional protease. However, this study cannot provide a conclusion as to this possibility. Both situations of protease activity in parasites have been reported. In *Leishmania donovani,* two proteases of identical molecular weight were purified; one was a metalloprotease that showed activity against collagen, while the other was a serine protease that preferentially degraded other synthetic substrates. It has been suggested that the latter protease may interfere in the process of phagocytosis (Choudhury *et al*., 2010). Meanwhile, in *Giardia intestinalis*, different activities for a single band have been reported previously, suggesting the possible existence of proteases with multiple functions (Williams & Coombs, 1995).

This work shows that the proteases of the ESP of *G. binucleatum*

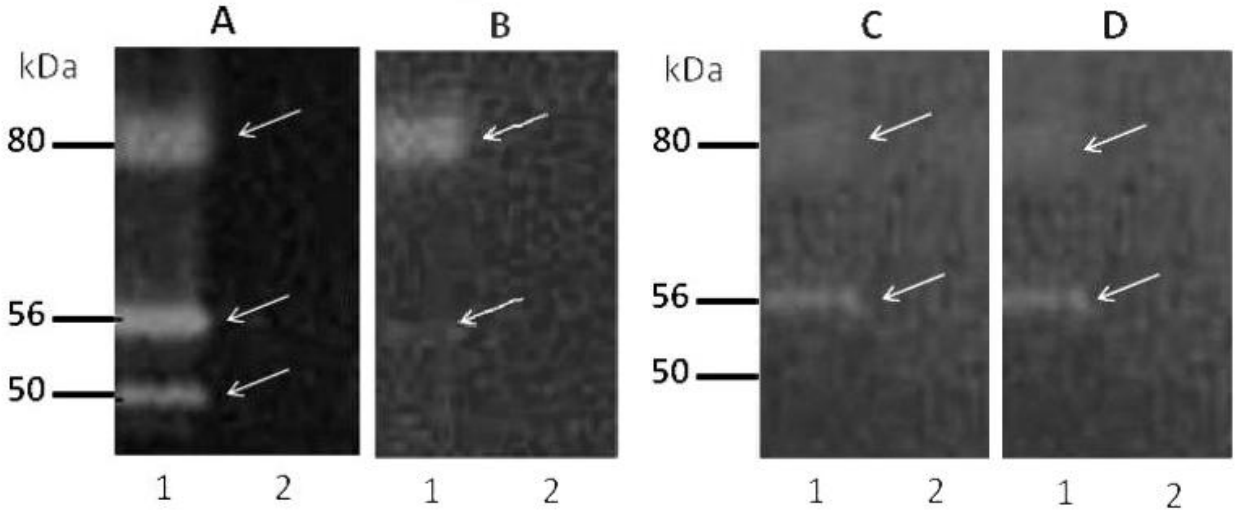

Fig. 2. Proteolytic activity of ES products of ADVL3 of *G. binucleatum* in polyacrylamide gels copolymerized with different substrates. (A) 005% bovine gelatin, (B) 0.0125% human fibronectin, (C) 0.025%, sheep gamma globulins, (D) 0.025% human gamma globulin. Lanes 1, 48-hour incubated ES products; Lanes 2, controls (PBS).

are capable of disrupting proteins in the extracellular matrix (fibronectin) and effectors of the host's humoral immune response (antibodies); therefore, they could be candidates for consideration as factors of pathogenicity in this parasite.

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