

Gnathostoma binucleatum: Excretion–secretion antigen analysis obtained from advanced third-stage larvae in in vitro culture

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Abstract

The paper describes an introductory characterization of antigenic stimulation of excretion–secretion products (ESP) of *Gnathostoma binucleatum* advanced third-stage larvae cultured in vitro and proteinases present in this products. Excretory and secretory proteins were obtained after 10 larvae were maintained in 5% CO₂ RPMI medium. The supernatant was collected each week for two months. The proteins were dialyzed, concentrated, and separated in 10% SDS–PAGE gels under reducing conditions and transferred to nitrocellulose paper for immunoblot analyses. *G. binucleatum* immunized mice serum was used to determine protein antigenicity. Four proteins of 40, 80, 120, and 208 kDa persisted for two months and three proteins, 80, 120, and 208 kDa were recognized for antibodies of mice. In SDS–PAGE gelatin substrate gels ESP resolved as two proteins with molecular weight of 80 and 208 kDa that were sensitive to a metalloproteinase inhibitor, and thus it may be inferred that they might be used for diagnosis of gnathostomiasis.

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Index Descriptors and Abbreviations: *Gnathostoma binucleatum*; Nematode; AdvL₃, advanced third-stage larvae; Protease; Cysteine protease; Serine protease; Metalloprotease; ESP, excretory–secretory products; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; IgG, immunoglobulin G; 1-*trans*-epoxy succinyl-leucylamide-4-guanidinol-butane (E64); Benzamidina; EDTA, ethylenediamine tetraacetic acid

1. Introduction

Gnathostomiasis is a systemic parasitic disease transmitted by raw or uncooked meat of intermediate hosts such as crustaceans and freshwater fish, ichthyophagous birds, amphibians, and reptiles that are infected with the advanced third-stage larva (AdvL₃) of the genus *Gnathostoma* which potentially may infect humans as an accidental intermediate host. We know that the disease

in humans is a combination of signs and symptoms caused by the parasite, such as mechanical damage due to the migration of the larvae, release of toxic substances, and the inflammatory reaction of the host (Miyazaki, 1960) the larvae can migrate to any part of the body and symptoms will depend on the affected site. This process can cause the “*larva migrans*” syndrome (Beaver, 1969) and symptoms can be cutaneous, ocular, neurologic or a combination of all. It has been reported that the cutaneous symptoms are the most prevalent in Mexico (Díaz-Camacho et al., 1998, 2003; Martínez-Cruz et al., 1989; Ogata et al., 1998; Pérez Polito et al., 1995; Rojas-Molina et al., 1999).

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The clinical diagnosis of gnathostomiasis is made with a positive biopsy for the parasite, however, it is difficult to find the larva because of its migratory condition.

The host-parasite interaction in human gnathostomiasis, especially concerning the immune response, is not completely understood. Serology has some limitations that prevent standardization, since antibodies for immunological diagnosis come from whole extracts of the parasite (Akao et al., 1989; Parkhouse and Harrison, 1989; Tapchaisri et al., 1991).

Improvements in immunological diagnosis of parasitic infections have focused on the study of excretion–secretion products (ESP). The specific functions attributed to the ESP of nematodes are invasion and migration through host tissue, facilitation of feeding and evasion of host immune responses (Tort et al., 1999). Nevertheless, ESP have been most valuable for diagnosis because they detect specific antibodies avoiding cross-reactions with other parasites. They have also helped to have a better understanding of the way parasites escape detection by the immune system. The identification of products secreted by nematodes suggests that they usually are a mix of single antigens compared to multiple antigens present in the raw extracts (Lightowlers and Rickard, 1988). But the biochemical mechanisms of tissue invasion by the infective larval stages of the genus *Gnathostoma*, as well as other *larva migrans*, are not well understood, although they appear to depend on parasite-derived proteases. As helminth larvae migrate through connective tissue, they release proteases that degrade extracellular matrix macromolecules in vitro and presumably function in histolysis (Hotez et al., 1990; McKerrow, 1989).

In this study, we investigate the excretion–secretion products of *Gnathostoma binucleatum* AdvL₃ and larval protease secretion using an in vitro model system towards their possible use in immune diagnosis.

2. Materials and methods

2.1. Parasite

The advanced third-stage larvae were collected from livers of experimentally infected mice at 18 post-infection day. The mice were previously infected orally with the early third-stage larvae (EaL₃) of *G. binucleatum* (Almeyda-Artigas, 1991) which developed in cyclops, as mentioned by Almeyda-Artigas et al. (1995). Briefly, the cyclops were infected experimentally with the second-stage larvae of the parasite obtained from eggs expelled from the uteri of a mature female dissected from a stomach tumour of a naturally infected dog from Tlacotalpan, Veracruz, Mexico (Colección Helminológica del Laboratorio de Sanidad Acuicola y Parasitología Molecular, UAM-Xochimilco catalog no. G638, 07/19/03).

2.2. Preparation of the excretion–secretion products

Gnathostoma binucleatum AdvL₃ were cultured in RPMI 1640 medium without supplement (Sigma, R0883) and prepared according to the manufacturer, containing 100 µg/ml penicillin (Sigma P7539) and 100 µg/ml streptomycin (Sigma P77539). The parasites were incubated for 24 h at 37 °C under 5% CO₂. They were maintained in the culture media for a week. Fifty milliliter conical tubes were used for collection changing the RPMI media every week. This procedure was performed throughout a month. Finally, the larvae were separated by centrifugation and the supernatant containing the excretion–secretion protein was dialyzed with distilled water overnight at 4 °C and concentrated to dryness by lyophilization and stored at –20 °C until needed. The lyophilized supernatants were resuspended in distilled water and used immediately or stored at –70 °C.

For substrate gel electrophoresis, lyophilized supernatants containing larval excretion–secretion were resuspended in 2 ml of Tris–HCl buffer 10 mM, pH 7.4, and AMICON UM-2 membrane filters were used for filtration and the content was used immediately. The total protein concentration was estimated by the Bradford Method (Bradford, 1976).

2.3. Immunized mice serum

Four 25 g Balb/c mice were injected in the peritoneum with 5 µg/mL of *G. binucleatum* larvae raw extract plus complete Freund's adjuvant v/v (100 µl) (F-5581). Eight days later the same amount of antigen plus incomplete Freund's adjuvant v/v (F-5560) were injected intradermally and repeated three more times, eight days apart, under the same conditions. Thus, the last immunization was given 30 days after the first one.

The mice were anesthetized and blood was drawn from the heart. Later they were killed. The serum was separated and kept at –20 °C until used.

2.4. Serum samples

Present investigation was carried out with 10 human normal sera controls and 10 human sera from patients with clinical manifestations suspected of having gnathostomiasis since they were found to have creeping eruption, migratory swellings, and history of eating raw meat of fishes. Diagnosis of one of the patients was made by a biopsy in which the larval gnathostomiasis was identified. Serum samples of 10 mice infected with *G. binucleatum* larvae were used. The mice were experimentally infected with larvae (AdvL₃) of the parasite and sacrificed 10 day post-infection. The liver of every mouse was analyzed and the parasites was identified for the confirmation of the infection.

2.5. Ten percent acrylamide/bisacrylamide gel electrophoresis

Forty micrograms of extracted proteins were separated by sodium dodecyl sulphate (SDS–PAGE) 10% acrylamide/bisacrylamide gel electrophoresis under reducing conditions at 100 V (Laemmli and Favre, 1973). The gels were stained with silver (Bio-Rad 161-0449) and Coomassie blue (Sigma B-0149).

2.6. Immunoblot

The electrophoresed proteins were transferred (“blotted”) onto a nitrocellulose membrane (0.2 µm Bio-Rad 162-0112) following Towbin’s method (Towbin et al., 1979), incubated in 5% Milk–PBS in phosphate buffer solution (PBS), and cut in 0.5 cm strips. Later they were incubated with human and mouse sera diluted 1:250 adding a peroxidase-labelled anti-mouse IgG conjugate (Sigma A-9040) diluted 1:1000. The strips were developed with 4-chloronaphthol (Sigma C-8890) in distilled water using H₂O₂ as substrate. The reaction stopped with distilled water.

2.7. Measurement of proteinase activity

Forty micrograms per milliliter of ESP were separated by sodium dodecyl sulphate polyacrylamide (SDS–PAGE) 12% acrylamide/bisacrylamide gel electrophoresis with 2%(w/v) of gelatin. Following electrophoresis at 200 V constant voltage (Laemmli and Favre, 1973). The gels were washed in three changes of 2–5% Triton X-100 over a 30 min period to remove the SDS and then incubated in appropriate buffer with proteinase inhibitor for 16 h at 37 °C (Olivos-García et al., 2003). Zones of proteolysis were visualized as clear bands against a blue background by Coomassie blue staining.

2.8. Characterization of proteinase using proteinase inhibitor

The proteinase inhibitor was applied at concentrations reported by Olivos-García et al. (2003). The inhibitors used were as follows: 20 µM (E-64) C₁₅H₂₇N₅O₅ (Sigma E3132) specificity for cysteine proteinase; Benzamide (Aldrich 13,582-8), specificity for the serine proteinase 10 mM EDTA for the metalloproteinases.

3. Results

3.1. Immunological characterization

Western blot analyses of ESP of parasite collected during two months with serum of mice immunized with *G. binucleatum* and serum of human infected with the

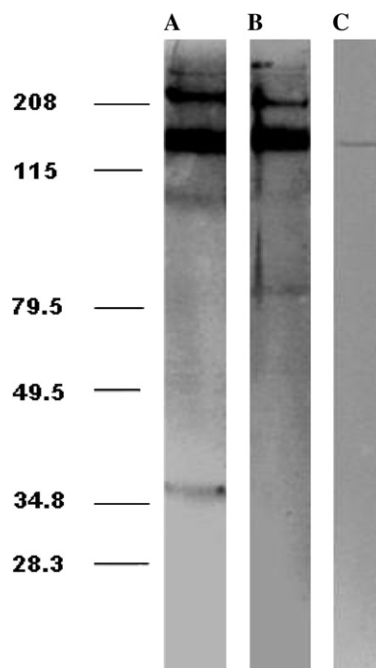


Fig. 1. Immunoblot with E–S of larvae (AdvL₃) and total extract of *G. binucleatum*. (A) Extracto total. (B) Excretory–secretory products with mouse serum. (C) Excretory–secretory products with human serum.

parasite, showed reactivity with three bands, 80, 120, and 208 kDa in the serum of mice and one protein of 120 kDa in the serum of human (Fig. 1). In comparison with the total extract of larvae of the parasite, the polyclonal antibodies of mice and human did not react to protein of 40 kDa, indicating that ESP of AdvL₃ has a high molecular weight in special for the protein of 120 kDa that was a recognized for the antibodies present in the serum of mice and human.

3.2. Proteolytic activity of ESP of *G. binucleatum* AdvL₃ in gelatin SDS–PAGE

Proteolytic products were detected in the culture medium. Analysis of proteinase-positive ESP of AdvL₃ in gelatin SDP-PAG clearly show two bands of proteinase activity at molecular weights of 80 and 208 kDa (Fig. 2).

3.3. Inhibitor assays

The effect of three differentiating proteinase inhibitors on ESP is shown in Table 1. Proteinase activity at two bands of 208 and 80 kDa were completely abolished by EDTA (metalloproteinase inhibitor) (Fig. 3).

4. Discussion

Excretion and secretion proteins from parasites are involved in certain metabolic activities of the parasite

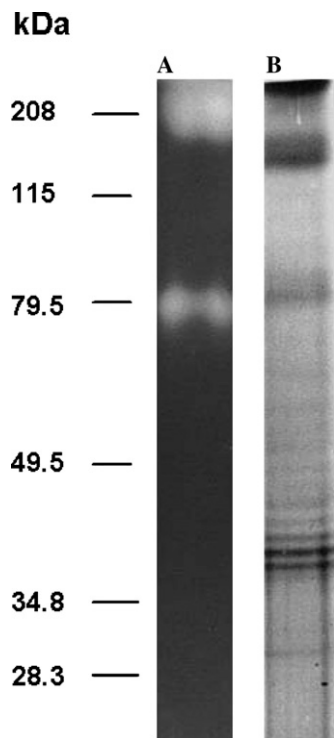


Fig. 2. Analysis of the proteinases activity in E–S of larvae (AdvL₃) of *G. binucleatum*. (A) Activity of E–S proteinases in larvae fractionated in gelatin-substrate gels 12%. (B) Analysis of E–S in larvae in polyacrylamide gel 10% staining with silver.

Table 1
Sensitivity of proteinases secreted by advanced third-stage larvae of *G. binucleatum* to inhibitors

Proteinase inhibitor	Proteolytic activity			
	208 (kDa)	120 (kDa)	80 (kDa)	35–40 (kDa)
No inhibitor	+	+	+	+
Cysteine	+	–	+	–
proteinase E-64	+	–	+	–
Serine proteinase	+	–	+	–
Benzamidina	–	–	–	–
Metallo	–	–	–	–
proteinase EDTA	–	–	–	–

and are essential in its pathogenic effects. In the case of *G. binucleatum* AdvL₃, it is assumed that E–S antigens are proteins produced during migration of the parasite. Probably toxic substances similar to acetylcholine or proteolytic enzymes such as hyaluronidase are also released. The larva, ingested in raw meat or insufficiently cooked freshwater fish is released in the host's stomach, perforates the intestinal mucosa, and migrates to the liver secreting some proteolytic enzymes and go to any part of the body causing the “*larva migrans*” syndrome. The secretion and excretion antigens could be modified according to the parasite needs, when it becomes mature. Thus, it is not surprising that some peptides might appear or disappear as shown in our results. Clearly excretion and secretion antigens must be the most sensi-

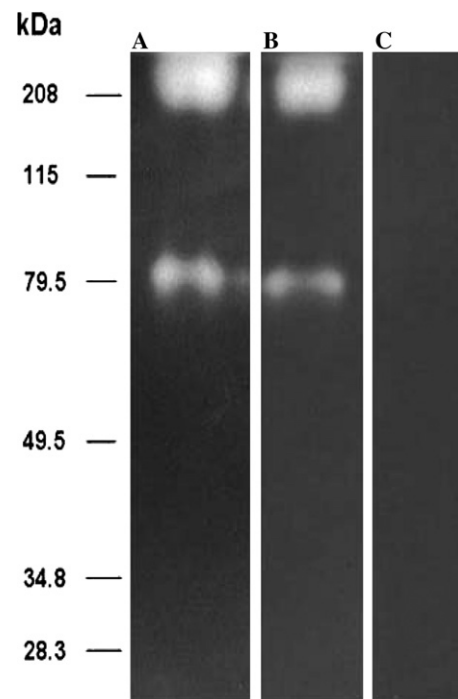


Fig. 3. The effect of a panel of class-differentiating proteinase inhibitors on E–S proteinases from larvae (AdvL₃) of *G. binucleatum*. (A) E-64. (B) Benzamidina. (C) EDTA. The final inhibitor concentrations used are listed in Section 2.

tive and specific for the diagnosis and evaluation of the effectiveness of chemotherapy of the disease, considering that they are the ones involved in the closest host–parasite interaction. As has been already mentioned, in the case of *Gnathostoma*, the displayed protease activity might be the most important, since it allows the parasite to travel through the skin as the infective stage or the filarial stage of the *Strongyloides stercoralis* does. It is not surprising that secretion antigens can reveal the parasite in much smaller concentrations of 0.35 µg in the intradermal reaction, as compared to antigens of the adult parasite of 4.0 µg (Neva et al., 2001). The excretion and secretion antigens of *Taenia solium* metacestodes are biologically so important that they have been used to establish the differences between active and inactive disease (Molinari et al., 2002), as well as to identify features responsible for the pathogenicity of the organisms. Excretion and secretion antigens with other parasites have been considered powerful antibody promoters proving their diagnostic usefulness in specificity and antigenicity (Assossou et al., 2004). Likewise they are also important for immune protection. For this reason the identification of the different components that induce antibody-mediated response during primary infection is most useful and could lead to a better understanding of immunity mechanisms in gnathostomiasis.

Gnathostoma binucleatum E–S antigens are especially significant for diagnosis because so far *Gnathostoma* species not found in Mexico have been used, such as *Gna-*

thostoma doloresi or *Gnathostoma spinigerum*, using adult forms for the antigen (Anantaphruti, 1989; Díaz-Camacho et al., 1998). Even though these species share antigens, adult forms are used instead of larvae which are the ones responsible for the disease. We are aware that adults can produce antigens because antigenic parasitic structures have been identified by immunofluorescence (Morakote et al., 1989), such as lateral cords, muscle pseudoceloma cuticle, and intestinal cells as the most immunogenic.

However, the *G. binucleatum* stage that infects humans is the larva which is responsible for the disease and the diagnosis should be made from its antigen since the adult form does not infect humans and might give a cross-reaction with other parasites. Therefore, excretion and secretion antigens are the most appropriate for know the physiological status of the worms (Martínez-Grueiro, 2002) and useful for diagnostic purposes.

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