An association between *Schistosoma mansoni* worms and an enzymatically-active protease/peptidase in mouse blood

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SUMMARY

An enzyme found previously in extracts of adult *Schistosoma mansoni* worms, that hydrolysed the chromogenic substrate N-acetyl-DL-phenylalanine β-naphthyl-ester, has here been further investigated and characterized. Evidence that the molecule found in the parasite was antigenically and enzymatically homologous with a constituent of normal mouse plasma has been consolidated using a monospecific serum in immunoelectrophoresis and Western immunoblotting. The molecular size of the enzyme was found to be approximately 70 kDa and it was inhibited by a serine protease inhibitor, but not by inhibitors of other classes of protease. The enzymatic activity found in normal mouse serum was also found in normal rat serum, but not in sera from several other mammalian species.

Key words: schistosome, *Schistosoma mansoni*, protease, esterase, naphthylamidase, mouse, rat, host, antigen, serum, immune evasion.

INTRODUCTION

An association between host-derived molecules and worms of the trematode *Schistosoma mansoni* has already often been demonstrated. The former include blood group (Smithers *et al*. 1969), Forssman (Dean and Sell, 1972) and major histocompatibility complex antigens (MHC: Gitter *et al*. 1982; Simpson *et al*. 1983), immunoglobulins (Kemp *et al*. 1977), complement decay-accelerating factor (Ramalho-Pinto, 1987), host lipids (Furlong *et al*. 1992), lipoproteins (Dinguirard and Yoshino, 2006), and the protease inhibitors alpha-2 macroglobulin (Damian *et al*. 1973) and contrapsin (Modha *et al*. 1988). Doenhoff *et al*. (1988) used immunochemistry and zymography to assay the peptidases present in different life-cycle stages of *S. mansoni* and found an enzyme which could hydrolyse N-acetyl-DL-phenylalanine β-naphthyl ester (NAPBNE) in extracts of adult worms. An apparently homologous antigen was, however, also present in normal mouse serum, suggesting that the molecule in the worms was of host origin.

That observation was novel on two counts: (i) the range of 'host antigens' associated with *S. mansoni* worms did not at that time include a protease/peptidase; and (ii) it is unusual to find a protease/peptidase that is present in mammalian serum in an enzymatically-active form as precedent would suggest that any proteases in plasma or serum would most likely circulate in pro-enzyme or zymogen form. The characterization of this protease/peptidase has here been taken further.

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MATERIALS AND METHODS

Parasite and antigens

A Puerto Rican isolate of *Schistosoma mansoni*, maintained by passage through random–bred mice and albino *Biomphalaria glabrata* snails, was used as the source of worm antigens. Mice were infected percutaneously with 200 cercariae and adult worms retrieved 42 days later by perfusion from an incised hepatic portal vein (Smithers and Terry, 1965), adapted as in Doenhoff *et al*. (1978). The worms were rinsed in perfusion fluid until all visible traces of erythrocyte contamination (and, it was assumed, all other host blood constituents) had been removed. Three different preparations of worm antigens were prepared as described previously by Doenhoff *et al*. (1988): (i) an aqueous extract of homogenized worms (WH); (ii) the supernatant containing antigens that were released by intact worms incubated at high density for 2 h in culture medium 199 (WS); and (iii) the antigens released from intact worms incubated in phosphate-buffered saline, pH 7·2, containing 2% deoxycholic acid (WM).

Rabbit antisera

A polyspecific rabbit antiserum was raised against normal mouse serum (Rα-NMS) by injecting the

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rabbit once-a-week subcutaneously with 1 ml of emulsion containing equal volumes of mouse serum and Freund's adjuvant (complete for the first injection and incomplete for the boosters).

The Rα-NMS reacted monospecifically in immunoelectrophoresis with a peptidolytic enzyme in extracts of adult *S. mansoni* worms. An antiserum that was relatively specific for the worm-derived antigen with peptidolytic activity was generated by immunizing another rabbit with replicates of the immunoprecipitin arcs containing the enzymatic activity that had been homogenized and emulsified in Freund's adjuvant (complete for the first injection and incomplete for the boosters), according to methods described previously (Goudie *et al.* 1966; Dunne *et al.* 1986).

Immunoelectrophoresis, immunoblotting and zymography

Adaptations of previously described methods were used for immunoectrophoresis (IEP: Williams and Grabar, 1955), adapted as in Dunne *et al.* (1986) and single radial immunodiffusion (RID: Mancini *et al.* 1965), adapted as in Darani *et al.* (1997). Both were carried out in 1 mm thick gel films of Noble agar on 84 mm × 84 mm glass plates in 0·06 M barbitone buffer, pH 8·6.

Peptidolytic activity on a chromogenic substrate was detected by zymography. Non-immunoprecipitated material in agar plates was removed by rinsing the films in several changes of excess isotonic saline for 24 h, followed by immersion in a substrate mixture consisting of 5 mg N-acetyl-DL-phenylalanine β-naphthyl-ester (NAPBNE, Sigma-Aldrich, Poole, Dorset, UK) and 10 mg fast blue B (FBB, purchased from Sigma-Aldrich), both of which had been dissolved in 2 ml of dimethyl formamide (DMF) and all of which was then diluted in 40 ml of 0·05 M phosphate-buffered saline (PBS), pH 7·6. The reaction was terminated when colour reactions were seen to have developed to an intensity that was appropriate for storage of the image as a photographic record.

One-dimensional electrophoresis in 12% polyacrylamide with sodium dodecylsulphate (SDS-PAGE) was performed as described by Studier (1973), adapted from Laemmli (1970) and Western immunoblotting was performed as described by Curtis *et al.* (1996), adapted from Towbin *et al.* (1979). Proteolytic enzyme activity in normal mouse serum was identified in 12·5% SDS-PAGE in which polypep (collagen polypeptides), haemoglobin, casein and elastin (all purchased from Sigma) had been incorporated into the acrylamide solution, all at a concentration of 2 mg/ml. The technique was an adaptation of the method described by Lockwood *et al.* (1987). The substrate gel was run under the same conditions as those for SDS-PAGE. After electrophoresis the gel was washed with 2·5% Triton X-100 to remove SDS and incubated overnight at 37 °C with appropriate buffer. The gel was subsequently stained with Coomassie blue and destained by rinsing in a mixture of ethanol: acetic acid: distilled water (30:7:63 by volume). The proteolytic activity was demonstrated as a clear band (i.e. where the substrate had been hydrolysed by the electrophoresed antigen) in a blue background.

In order to determine the class of peptidase in mouse plasma, the enzyme was displayed by immunoprecipitation with antiserum in radial immunodiffusion. After removal of non-precipitated serum and other constituents by washing in excess saline, individual rings of immunoprecipitate were incubated in a solution of a protease inhibitor, followed by washing and staining with the chromogenic substrate (NAPBNE and fast blue B). The rings were then photographed over direct light. The protease inhibitors were p-hydroxy mercuribenzoic acid, phenylmethylsulphonyl fluoride (PMSF), N-tosyl-phenylalanine chloromethyl ketone (TPCK); N-p-tosyl-L-lysine chloromethyl ketone (TLCK), phenanthroline, soya bean trypsin/chymotrypsin inhibitor and soya bean trypsin inhibitor, all purchased from Sigma.

RESULTS

Fig. 1A and B show that a monospecific rabbit serum that had been raised against the peptidase present in *S. mansoni* worms also reacted in immunoelectrophoresis with an antigen with similar enzymatic activity in normal mouse serum (NMS). The cathodal tips of the enzymatically-active precipitin arcs produced respectively by the polyspecific and monospecific antisera gave a 'line of identity', indicating that the two sera were precipitating the same antigen.

Fig. 1C and D show the reactivity of the rabbit anti-normal mouse serum antiserum in immunoelectrophorisis against different preparations of adult *S. mansoni* worm antigens. Enzymatically-active precipitin arcs were produced from the worm homogenate (WH) and more intensely from the deoxycholic acid extract of the worms (WM), but not from worm culture supernatant (WS). The rate of migration of the enzyme in the worm preparations was similar to that of the molecule reactive with the same substrate in NMS (Fig. 1D).

Zymography with the same chromogenic substrate applied to normal mouse serum proteins separated by electrophoresis in a polyacrylamide gel indicated there was 1 peptidase in NMS that had a relative molecular size of approximately 70 kDa (Fig. 2A). This estimate of the size of the enzyme was supported by the results of a Western immunoblot of WM (doexycholic extract) of *S. mansoni* and of NMS

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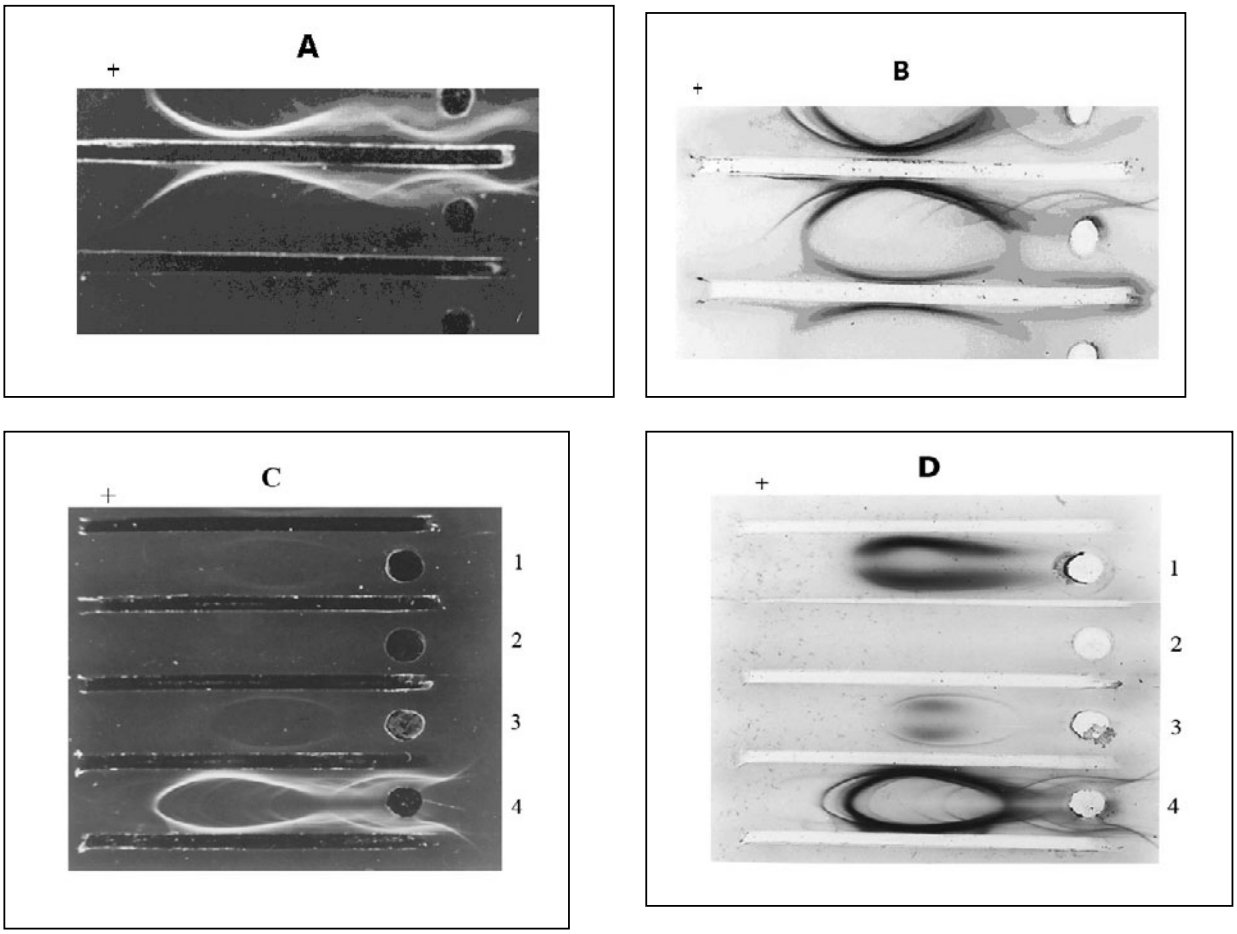

Fig. 1. (A and B) Immunoelectrophoresis and zymography of normal mouse serum (NMS). All three antigen wells contained 10 μl of NMS which was electrophoresed. The upper serum trough contained 100 μl of a polyspecific rabbit anti-whole normal mouse serum (Rα-NMS) and the lower trough 100 μl a rabbit serum raised against immunoprecipitin arcs containing a *Schistosoma mansoni* worm antigen with naphthylamidase activity. The plate was photographed over indirect lighting before staining (A), or over direct lighting after zymographic staining with the chromogenic substrate mixture of NAPBNE and FBB (B). The most anodal antigen precipitated by the polyspecific anti-NMS is assumed to be mouse serum albumin. (C and D) Immunoelectrophoresis of Rα-NMS (in all 5 serum troughs) reacting against *S. mansoni* WM (antigen well 1), WS (2) WH (3) and NMS (4). The plate was photographed over indirect lighting before staining (C) or over direct lighting after zymographic staining with the chromogenic substrate mixture of NAPBNE and FBB (D).

probed with the monospecific rabbit antiserum, as the principal antigen detected by this serum had a molecular weight of approximately 70 kDa in both worm doexycholic extract and normal mouse serum (Fig. 2B).

To investigate whether the enzyme had proteolytic (as well as peptidolytic) activity, and if so, to assay for its substrate specificity, zymography was performed in SDS-PAGE gels in which different protein substrates had been incorporated. The substrates tested included collagen-derived peptides, haemoglobin, casein and elastin. Results of this test showed that normal mouse serum gave a band of proteolytic hydrolysis at ~70 kDa in the gel with the collagen-derived substrate. No proteolytic activity was found on the other proteinaceous substrates tested (not shown).

To determine the type of protease in normal mouse serum the enzyme was immunoprecipitated by radial immunodiffusion and individual circles of immunoprecipitate were then incubated in solutions of different protease inhibitors. Staining with the chromogenic substrate mixture showed the enzymatic activity to have been inhibited by PMSF and TLCK, only partially inhibited by TPCK, and uninhibited by hydroxymercuribenzoic acid, phenanthroline or either of 2 soya bean protease inhibitors (Fig. 3).

Fig. 4 shows that, as well as being present in mouse serum, a band of naphthylamidase activity of ~70 kDa was found in rat serum, but not in sera from a Syrian hamster, rabbit, guinea pig, sheep, cow or human. Rat serum (Fig. 4, lane 3) gave a somewhat greater intensity of staining than the same amount of mouse serum (Fig. 4, lane 1). There was, however, no apparent difference between mouse serum and mouse plasma in the intensity of staining at ~70 kDa (result not shown.)

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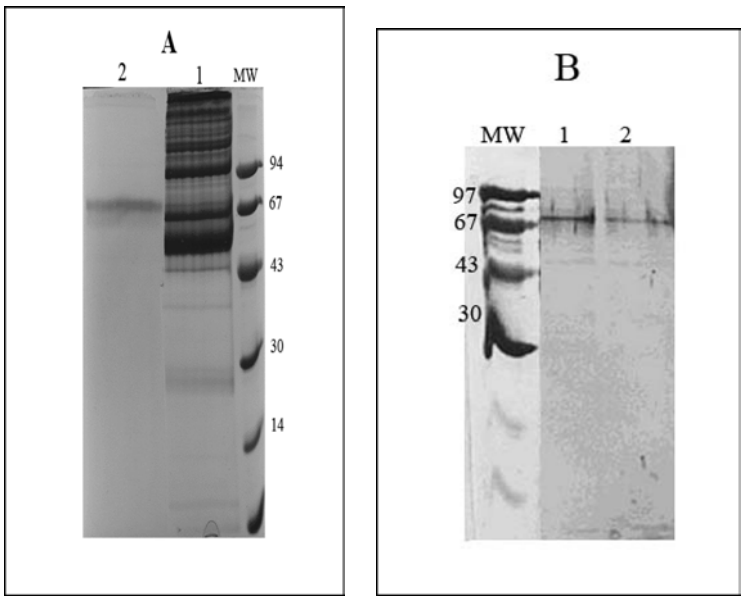

Fig. 2. Electrophoresis and immunoblotting of normal mouse serum and *Schistosoma mansoni* worm antigens in SDS-PAGE. (A) SDS-PAGE of 5 μl of NMS stained with chromogenic substrate mixture NAPBNE + FBB (Lane 2) or Coomassie blue (Lane 1). MW: molecular weight markers. (B) Western immunoblot of *S. mansoni* WM (Lane 1) or NMS (Lane 2) probed with the monospecific antiserum raised against immunoprecipitin arcs containing an *S. mansoni* worm antigen with naphthylamidase activity. MW: molecular weight markers.

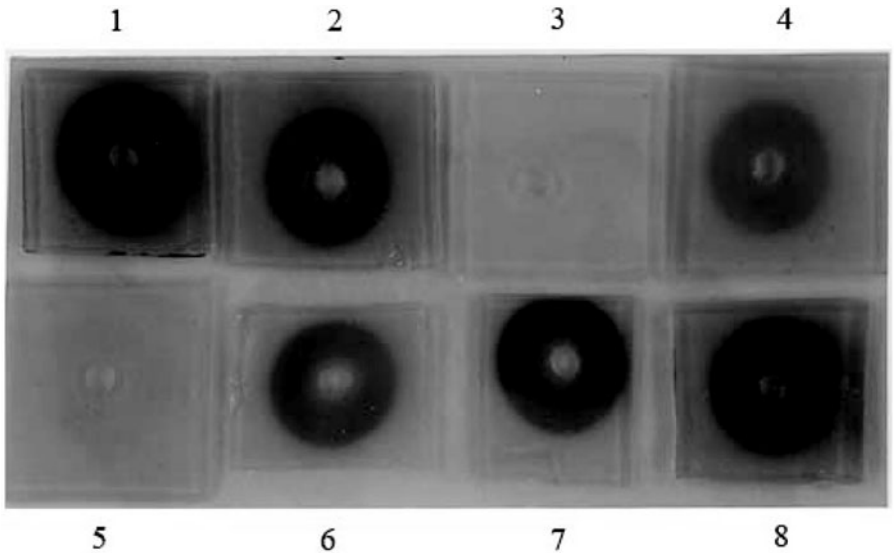

Fig. 3. Effect of protease inhibitors on naphthylamidase activity immunoprecipitated from normal mouse serum by RID (8 μl of 1/6 diluted NMS in each well and 0·3 ml of Rα-NMS in 10 ml gel). The protease inhibitors were as follows: (1) control – no inhibitor; (2) 10 mM p-hydroxy mercuribenzoic acid; (3) 10 mM phenylmethylsulphonyl fluoride (PMSF); (4) 10 mM N-tosyl-phenylalanine chloromethyl ketone (TPCK); (5) 10 mM N-p-,tosyl-L-lysine chloromethyl ketone (TLCK); (6) 10 mM phenanthroline; (7) 2 mg/ml soya bean trypsin/chymotrypsin inhibitor; (8) 2 mg/ml soya bean trypsin inhibitor.

DISCUSSION

We have shown that a monospecific serum that had been raised against an antigen with naphthylamidase activity present in extracts of adult *S. mansoni* worms also reacted with an antigen with the same enzymatic activity and relative molecular size in normal mouse serum. Serum from rats was found to have a similar enzymatic activity, but this activity was not found in sera from several other animal species. The respective forms of the enzymatically-active molecule in the worm and mouse serum appeared to be antigenically identical and the results are consistent with this molecule being a host-derived antigen

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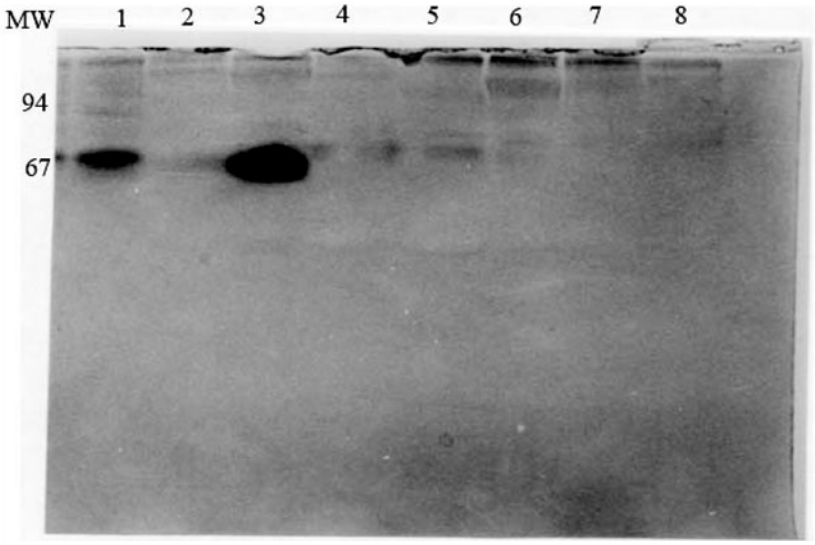

Fig. 4. SDS-PAGE of sera from different animal species subjected to zymography with substrate mixture NAPBNE + FBB. The electrophoresed sera were: (1) mouse; (2) hamster; (3) rat; (4) guinea pig; (5) rabbit; (6) sheep; (7) bovine; (8) human. MW = molecular weight indicators.

associating with the detergent-soluble, aqueous insoluble elements of this parasite.

A band of hydrolysis of collagen-derived peptides was found at approximately the same molecular size as the naphthylamidase activity in a protease substrate gel indicating the enzyme could be a protease or peptidase. It could be present in the schistosome worms simply as a contaminant of extracts of worms that have not been adequately separated from constituents of the blood in which they had been living but this seems unlikely as the polyspecific rabbit α-NMS did not appear to detect host albumin, a more abundant host serum protein, in the parasite antigen preparations. In Fig. 1C the rabbit anti-NMS can, however, be seen to have precipitated another molecule without any enzymatic activity from WM and WH and this antigen may be contrapsin, another host-derived molecule that is known to be present in *S. mansoni* worms (Modha *et al.* 1988).

The enzyme under investigation here (derived from mouse serum) was inhibited by PMSF which is consistent with it being a serine protease. Throughout this work it was identified through its ability to hydrolyse N-acetyl-DL-phenylalanine naphthyl ester, suggesting it has chymotrypsin-like activity. Paradoxically, however, it was not fully inhibited by TPCK, a conventional inhibitor of chymotrypsin, but was inhibited by TLCK, an inhibitor of trypsin. We do not have an explanation for the discrepancy between the substrate and inhibitor profiles of the enzyme.

It is perhaps surprising that the enzyme was found circulating in an apparently active form as proteases in mammalian bloods are generally present as pro-enzymes or zymogens. There was no difference between mouse blood and plasma with regard to detection of the enzyme (result not shown) indicating that the enzyme was not generated as a result of the coagulation cascade having been activated. It was, however, only found in mouse and rat blood, a result that would seem to have no bearing on the relative permissiveness of the respective hosts to *S. mansoni* infection (Cioli *et al.* 1977), mice, hamsters and humans being considered permissive, while rats, rabbits, and the other species are relatively non-permissive.

Our estimates of the relative size of the enzyme ($\sim$70 kDa) unfortunately reveal little about the identity of the enzyme since it is in the size range of both the inactive precursors and active forms of the enzymes involved in blood coagulation and fibrinolysis e.g., 72 kDa pro-thrombin and 75 kDa plasmin.

Rabbit anti-NMS precipitated a more intense level of enzymatic activity from deoxycholate extracts of worms than from either whole worm homogenate or worm supernatant (Fig. 1D). Deoxycholic acid is a detergent that disrupts membranes by intercalating into phospholipid bilayers, thus solubilizing both lipids and membrane-bound proteins. This result suggests that although the enzyme has a similar relative molecular size whether in blood or in the parasite, it may be membrane-bound in the latter. We have no obvious explanation for why the form of the enzyme associated with schistosome worms seems to be detergent extractable, while that in host blood seems to be aqueous soluble.

The use made of the host–derived protease by the parasite can only be speculated. It may be part of the immune-evasion repertoire of host antigens (Maizels *et al.* 1993). Consistent with this possibility, we found that in indirect immunofluorescence the enzyme was found on the surface of 6-day-old

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mouse lung-derived schistosomula, but not on the surface of mechanically-transformed schistosomula (H. Y. Darani, unpublished results). Alternatively, after uptake the enzymatic activity of this molecule may be exploited in some way by the parasite. Further work is thus needed to investigate these possibilities and to characterize the molecule, particularly with regard to its amino acid sequence and relationship to other serine proteases.

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