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Trichinella spiralis Infective L_1 Larvae Secrete Serine Proteinase(s)

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Abstract

Trichinella spiralis infective L_1 larvae secreted one or more proteinases during *in vitro* culture. The enzyme(s) degraded azocasein, azocoll and elastin-orcein optimally at pH 8.8, and hydrolyzed succinyl-$(Ala)_3$ *p*-nitroanilide at this pH. Assays using protease inhibitors showed that phenylmethanesulfonyl fluoride decreased the enzyme activity. Electrophoresis on sodium dodecyl sulfate-polyacrylamide gel copolymerized with gelatin exhibited the major activity at 150 kDa and a minor activity at 98 kDa. These results suggest that *T. spiralis* infective larvae produce serine proteinase(s) which exhibit the characteristics of elastase and molecular weights of 150,000 and 98,000.

Key words: *Trichinella spiralis*, ES products, serine proteinase, elastase

Introduction

Parasitic helminths modulate the host response by producing many kinds of proteins with various biological activities. One major group of products includes proteinases produced by many species of nematodes (Sakanari, 1990) and trematodes such as *Schistosoma mansoni* (McKerrow *et al.*, 1985) and *Fasciola hepatica* (Rupova and Keilova, 1979). They appear to play roles in penetration and migration into the host tissues, blood feeding, and other biological events.

Trichinella spiralis also produces many proteins at each stage of its life cycle (Almond *et al.*, 1986). Some of them have been characterized as superoxide dismutase (Rhoads, 1983) and major antigens (Denkers *et al.*, 1990; Gold *et al.*, 1990; Sugane and Matsuura, 1990; Sugane *et al.*, 1987; Vassilatis *et al.*, 1992; Zarlenga and Gamble, 1990). However, there have been no reports on the proteolytic enzymes of *T. spiralis*. Infective L_1 larvae of *T. spiralis* penetrate the villus of the small intestine after they have been liberated by the action of digestive enzymes of the host and transferred to the small intestine. Newborn L_1 larvae penetrate the small intestine into the mesenteric lymphatics or bloodstream, pass through host tissues, and finally invade into skeletal muscles to form nurse cells. We supposed that proteinases secreted by *T. spiralis* larvae might be concerned in some of these biological events and so searched for proteinases in the excretory-secretory (ES) products. In this paper, we documented that *T. spiralis* infective larvae produce serine proteinase(s) which exhibit the characteristics of elastase and molecular weights of 150,000 and 98,000.

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Materials and Methods

ES products of T. spiralis *larvae*

Muscle larvae were recovered from infected ddY mice by peptic digestion as described by Rhoads *et al.* (1983). The larvae were cultured in RPMI 1640 medium in the presence of penicillin (100 IU/ml), streptomycin (100 μg/ml) and amphotericin B (0.25 μg/ml). The medium was changed every two days for 6 days and the collected supernatant was stored at 4°C. The pooled supernatant was concentrated by ultrafiltration in an Advantec stirred cell using an UK-10 ultrafilter (Advantec, Japan; exclusion range 10,000 mol. wt.). The concentrated ES products were dialyzed against distilled water. Aliquots were lyophilized and stored at –20°C until use.

Enzyme activity and its inhibition profiles

All the substrates and protease inhibitors used were purchased from Sigma Chemical Co. (USA).

Aliquots of ES products (ca. 1.5 mg) were incubated with the proteolytic substrates (final concentration: 5 mg/ml) in the buffers of the appropriate pH. The buffers used for the assay were as follows: 0.25 M citrate buffer (pH 4.0–6.0), 0.25 M phosphate buffer (pH 6.0 and 7.2), 0.25 M Tris-HCl buffer (pH 7.2–8.8), and 0.25 M carbonate-bicarbonate buffer (pH 8.8–11.0). Undigested proteins were removed by acid-precipitation (azocasein) or centrifugation (azocoll and elastin-orcein), and the absorbance of the supernatants was measured at 440 nm (azocasein), 520 nm (azocoll) or 550 nm (elastin-orcein).

Synthetic substrates were dissolved at 1 mg/ml in dimethylsulfoxide. The reaction mixture was composed of 10 µl ES product solution (15 mg/ml), 70 µl 0.5 M Tris-HCl buffer (pH 8.8), and 20 µl substrate solution. The initial and final absorbances were measured at 405 nm. All activities were corrected for non-enzymic hydrolysis by subtraction of the appropriate reagent blank.

Proteinase inhibition assays were performed at the optimum pH after preincubation of ES products with various inhibitors at 4°C for 30 min. The concentrations of inhibitors are shown in Table 1.

Gelatin sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The detection of proteinase activity by electrophoresis on SDS-polyacrylamide gels copolymerized with gelatin was performed as described by McKerrow *et al.* (1985). An 8% (w/v) polyacrylamide gel was polymerized with 0.2% (w/v) bacto gelatin (Difco Lab., USA). ES products were dissolved in sample buffer without reducing agents and electrophoresed with cooling. After electrophoresis, the gel was washed with 100 mM Tris-HCl buffer (pH 8.8) for 30 min at room temperature and incubated with the same buffer overnight at 37°C. Then the gel was fixed and stained with 0.1% (w/v) Coomassie Brilliant Blue R-250 in 25% methanol/10% acetic acid (v/v), and proteolytic activities appeared as clear bands on a blue background. For calculation of molecular weight, standard molecular weight markers were run simultaneously.

Results

pH optimum for proteinase activity

The preliminary study showed that ES products had an azocasein-degrading activity, and that more than 60% of the activity was secreted in first 2 days (data not shown). We checked the pH optimum for the proteinase activity using azocasein as a substrate. The activity was detected in a broad range of pH from 5.0 to 10.0 with a peak at pH 8.8. A similar profile was obtained using azocoll and elastin-orcein (Fig. 1).

Effect of protease inhibitors

The effects of various protease inhibitors on azocasein degradation are shown in Table 1. Of the 8 inhibitors used, only PMSF (1 mM) decreased the total activity by 60%. DTT, a thiol proteinase activator, did not increase, but decreased the activity (Table 1).

Proteinase activities on synthetic substrates

To determine whether or not the proteolytic activities detectable with azocasein were effective for synthetic substrates, we incubated ES products with BAPNA, Suc-Phe-pNA, Suc-$(Ala)_3$-pNA or Leu-pNA, which is a substrate specific for trypsin, chymotrypsin, elastase, or leucine aminopeptidase, respectively. ES products hydrolyzed Suc-$(Ala)_3$-

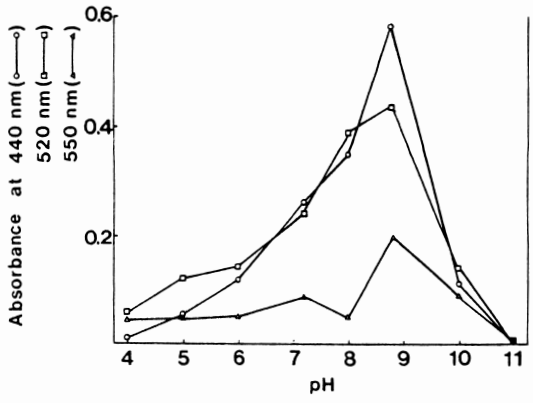

Fig. 1 Influence of pH on dye release after 2 h incubation at 37°C from azocasein (○—○), azocoll (□—□) and elastin-orcein (△—△) at a concentration of 5 mg/ml.

Table 1 Effect of protease inhibitors on azocasein-degrading activity of ES products

Inhibitor	Concentration	% Activity
none		100
ethylenediaminetetraacetic acid (EDTA)	(5mM)	102
phenylmethanesulfonyl fluoride (PMSF)	(1mM)	39
N-ethylmaleimide (NEM)	(2mM)	88
pepstatin	(10 μg/ml)	91
elastatinal	(0.1mg/ml)	96
leupeptin	(0.1mg/ml)	98
tosyllysine chloromethyl ketone (TLCK)	(10 μg/ml)	105
tosylphenylalanine chloromethyl ketone (TPCK)	(10 μg/ml)	94
dithiothreitol (DTT)	(1mM)	34

pNA. Release of pNA from Leu-pNA was less than 20% of that from Suc-$(Ala)_3$-pNA (Table 2).

Gelatin SDS-PAGE

Since ES products degraded denatured collagen (azocoll), SDS-polyacrylamide gel polymerized with gelatin was used to detect the bands with proteinase activity. Although ES products contained many proteins (Fig. 2, lane A), only two bands showed proteinase activity in gelatin-copolymerized gel (lane B). A band with the major activity appeared at 150 kDa. A minor activity was detected at 98 kDa.

Table 2 Nitroanilide released from various substrates by ES products

Substrate	Nitroanilide released*
BAPNA‡	UD†
Suc§-Phe-pNA‖	UD
Suc-$(Ala)_3$-pNA	0.140
Leu-pNA	0.026

* Absorbance at 405 nm after incubation for 24 h at 37°C.
† undetectable
‡ *Nα*-benzoylarginine *p*-nitroanilide
§ succinyl
‖ *p*-nitroanilide

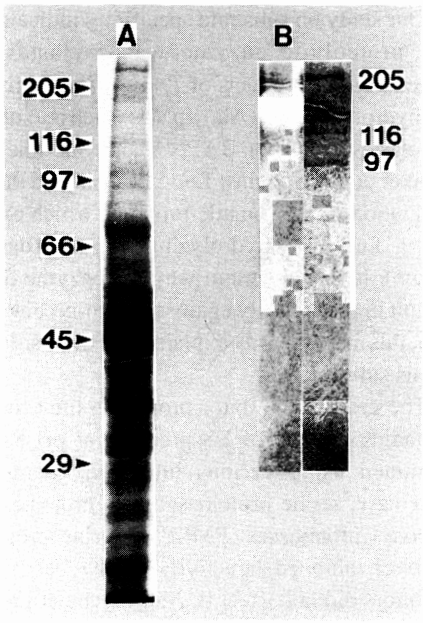

Fig. 2 (A) SDS-PAGE of ES products from infective L_1 larvae of *T. spiralis*. (B) Gelatin SDS-PAGE of ES products from infective L_1 larvae of *T. spiralis*. Eight-percent polyacrylamide gels were polymerized with 0.2% bacto gelatin. ES products were not boiled and reducing agents were not used in A and B.

Discussion

We report here for the first time that ES products of *T. spiralis* infective L_1 larvae exhibited proteolytic activities with alkaline pH optima, substrate specificities and PMSF-sensitivity.

The ES products degraded azocasein in a pH 5.0–10.0 range with the optimal enzyme activity at pH 8.8. The pH-dependency was also investigated with azocoll and elastin-orcein, although less pigment was released than from azocasein. The alkaline pH optimum has been reported for secreted proteinases from other helminths including *Toxocara canis* (Robertson *et al.*, 1989), *Ascaris suum* (Knox and Kennedy, 1988), *Ancylostoma caninum* (Hotez *et al.*, 1985), *Necator americanus* (Matthews, 1982) and *S. mansoni* (McKerrow *et al.*, 1985). They possess characteristics of serine proteinases and are thought to be concerned in the penetration of parasites into the host.

Our study on substrate specificity indicated that this proteolytic enzyme was a elastase-like proteinase. ES products of *T. spiralis* infective larvae hydrolyzed Suc-$(Ala)_3$-pNA which is a substrate for elastase, but not BAPNA and Suc-Phe-pNA. Weaker activity against Leu-pNA may be the contamination of the somatic proteins, which exhibits strong Leu-pNA hydrolyzing activity (data not shown). It was uncertain why the enzyme did not exhibit higher activity against elastin-orcein. However, this may be partly explained by the insolubility of this substrate.

The protease inhibitor profile on the azocasein degrading activity of ES products at pH 8.8 was examined using various inhibitors to metalloproteinase, serine proteinase, thiol proteinase and carboxyl proteinases. PMSF, a serine proteinase inhibitor, inhibited the activity by 60%, but the other inhibitors did not affect it. Neither chelating agent (EDTA) nor a thiol-inactivator (NEM) decreased the degradation of azocasein. Therefore, the proteinase activity did not depend on bivalent cations or thiol-groups. In addition, thiol-activator (DTT) did not increase but rather decreased the degradation, indicating that the S-S bondage in the proteinase molecule may be needed to express the activity. Pepstatin, a carboxyl proteinase inhibitor, also did not inactivate the enzyme. These results suggest that the ES products may contain one or more serine proteinases. Furthermore, elastatinal (an elastase inhibitor) and the other inhibitors of some serine proteinases, leupeptin, TLCK and TPCK, had no inhibitory activity. These inhibition profile of the enzyme together with its substrate specificity are similar to that of rat leucocyte elastase (RLE) (Gardi *et al.*, 1991). RLE has been reported to hydrolyze elastin and synthetic substrates for elastase such as Suc-$(Ala)_3$-pNA, and to be insensitive to elastatinal.

The molecular weight of the enzyme was examined using an SDS-polyacrylamide gel polymerized with 0.2% gelatin. Electrophoresis and incubation at 37°C followed by protein-staining visualized gelatin-degrading activities at 150 kDa and 98 kDa. The relation between the main band at 150 kDa and the faint band at 98 kDa is unclear. The size of this proteinase is unexpectedly large compared with the reported helminthic proteinases except two, 120 kDa enzyme of *T. canis* (Robertson *et al.*, 1989) and 170 kDa enzyme of *Dirofilaria immitis* (Swamy and Jaffe, 1983). The precise molecular weight(s) of the enzyme(s) and the relation of two bands at 150 kDa and 98 kDa will be elucidated by purification or gene cloning of the enzyme.

Many kinds of proteinases have been demonstrated in ES products of various helminths. *S. mansoni* cercariae (McKerrow *et al.*, 1985) produce a proteinase which is believed to play a role in penetrating the host skin. A similar role of proteinases from infective larvae was proposed for *N. americanus* (Matthews, 1982) and *Strongyloides* spp. (Rege and Dresden, 1987). Both microfilariae and L_3 larvae of *Brugia malayi* and some species of *Onchocerca* (Petralanda *et al.*, 1986; Lackey *et al.*, 1989) produce proteinases that may facilitate the migration through cutaneous tissue. Another proteinase with the biological activity of hydrolyzing a number of plasma proteins was reported in *A. caninum* (Hotez *et al.*, 1985). Hemoglobinase activity was detected in adult somatic extracts of *S. mansoni, D. immitis, Angiostrongylus cantonensis, A. suum* (Maki and Yanagisawa, 1986), and *F. hepatica* (Rupova and Keilova, 1979). A proteinase of *Haemonchus contortus* mediates the ecdysis of the infective larvae (Gamble *et al.*, 1989). Others are produced by infective larvae of *Anisakis simplex* (Kennedy *et al.*, 1988), *A. suum* (Knox and Kennedy, 1988) and *T. canis* (Robertson *et al.*, 1989), and involved in tissue invasion.

Possible biological function of the proteinase in *T. spiralis* is thought to concern the penetration in the host tissues. Infective L_1 larvae are excysted and live in the walls of the small intestine and grow to adults. Newborn L_1 larvae penetrate the small intestine and invade many tissues after migration through the bloodstream. Finally they invade skeletal muscles and form nurse cells. At each stage the worm invades host tissues, and it is intriguing to consider that the proteinase reported here may be concerned in the degradation of the host tissues or cells.

Another possibility is that the enzyme plays a role in forming nurse cells. The larvae change the biological nature of the host muscle cells and secreted antigens are present in the nuclei or cytoplasm of nurse cells (Lee *et al.*, 1991; Vassilatis *et al.*, 1992). Furthermore, ES products induce the reorganization of the host muscle cells and nuclear

divisions (Ko *et al.*, 1992). Some enzymic or signaling proteins might be secreted by the larvae. These biological activities of the enzyme should be further studied.

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