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Proteolytic enzymes secreted by larval stage of the parasitic nematode *Trichinella spiralis*

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Abstract. Excretory/secretory products (ES), collected from *in vitro* cultures of muscle larvae (L1) of *Trichinella spiralis* (Owen, 1835) were examined for the presence of proteolytic enzymes. Several discrete proteinases in the size range of 25-55 kDa were identified by substrate gel electrophoresis and were characterised according to pH optima, substrate specificity and inhibitor sensitivity using azocasein assay. Serine, cysteine and metalloproteinases active at pH 5-7 were identified. The serine proteinases were found to predominate and some of them were found to be specific for the larval stage of the parasite. The results from the substrate analysis indicated the presence of collagenolytic and elastolytic activities. The proteinase activity was inhibited by IgG isolated from *T. spiralis*-infected mice, an observation of relevance to understanding host/parasite interactions and, ultimately, the development of anti-*Trichinella* vaccine.

Proteolytic enzymes are receiving increasing attention as biologically important molecules for parasites in a range of biological processes. They are presumably involved in processes such as penetration of host tissues, parasite nutrition, anti-coagulation and evasion of host immune responses (McKerrow 1989). It is now clear that parasite enzymes can stimulate host protective immunity and may be potent allergens (Stromberg 1980, Kennedy et al. 1991, McKeand and Knox 1994).

Trichinella spiralis (Owen, 1835) is a parasitic nematode which is capable of infecting a wide variety of mammals including humans. The complete life cycle of the parasite occurs in a single host and begins when the host consumes infected meat. The life cycle of *Trichinella* has been well characterised (Despommier 1983) and comprises the adult stage in the host gastro-intestinal tract, a migratory phase during which the newborn larvae pass through the blood and lymphatics to the skeletal muscle cells, and encapsulated larvae (L1 larva) in the muscles which represent the infective stage of the parasite. The infective first-stage larva occupies the striated muscle cells, which are radically altered as a result of the infection. The mechanisms by which the larvae penetrate tissues are unknown, although the importance of proteolytic enzymes is suspected (McKerrow 1989, Knox 1994).

Previous investigations showed that the adult *Trichinella spiralis* secrete *in vitro* a number of proteinases (Todorova et al. 1995) with optimal activity at pH 7.5. Serine proteinases predominated and were found to be targets of an antibody response.

The present study concentrates on the proteolytic activity of excretory/secretory products (ES) of *T. spiralis* larvae. The pH optimum, substrate specificity, inhibitor sensitivity and the effect of antibody, isolated from immune animals, on proteolytic activity were studied.

MATERIALS AND METHODS

Parasites and preparation of ES. *Trichinella spiralis* larvae (L1) were isolated by the standard pepsin digestion method (Brand et al. 1952) from the muscles of 10-12-week-old mice, which had been orally infected 28 days previously. Adult parasites were isolated from intestines of mice infected 5 days previously as described elsewhere (Todorova et al. 1995). The parasites were extensively washed in Hank's balanced salt solution (HBSS, Gibco Ltd), supplemented with antibiotics as described before (Todorova et al. 1995). The parasites were maintained for 3-4 days at 37°C, 5% CO_2 at a concentration of 10 000 larvae in 10 ml and 100 adults/10 ml of RPMI 1640, Dutch modification (Gibco Ltd), containing the following additives: 2 mM L-glutamine; 1 mg/ml glucose; 400 ng/ml glycyl-1-histidyl-1-lysine; 40 μg/ml glutathione; 5 μg/ml Fungison; 100 IU/ml penicillin; 100 μg/ml streptomycin; 25 μg/ml gentamycin sulphate and 1 mM sodium pyruvate. Culture supernatants were concentrated, dialysed and stored at -20°C. The protein concentration, estimated using a Coomassie Blue-based assay (Pierce Chemical Co), was typically between 70 and 100 μg/ml.

Effect of pH on enzyme activity. The effect of pH on activity against azocasein was tested over the range pH 4-9, using 0.1 M acetate buffer (pH 4-6), 0.1 M phosphate buffer (pH 5-7) and 0.1 M Tris-Cl buffer (pH 7-9). The assay was performed in triplicate.

Enzyme assays. Proteolytic activity was measured spectrophotometrically by use of chromogenic substrates azocasein, azocoll, and elastin-orcein (Sigma Chemicals Co.) as follows: the ES samples (20 μl) were mixed with 5 μl

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substrate (5 mg/ml) and 100 µl incubation buffer (0.1 M citrate/phosphate buffer, pH 5 or 1M Tris-Cl buffer, pH 6, or 0.1 M Tris-Cl, pH 7) and incubated for 16 hours at 37°C. After precipitation with equal volume of 1M perchloric acid (for azocasein) or centrifugation (for azocoll and elastin-orcein) the absorbance was read at 405 nm (azocasein) or 540 nm (azocoll and elastin-orcein). Activities were corrected for non-enzymic hydrolysis by subtraction of the appropriate reagent blank.

Polyacrylamide-substrate gels. Protease activity was monitored also on SDS gels. The procedure was described elsewhere (Todorova et al. 1995). Briefly, 20 µl sample diluted with 15 µl sample buffer (5% SDS, 20% glycerol, 0.01% bromophenol blue in 0.5 M Tris, pH 7) was electrophoresed on 10% or 5-25% polyacrylamide gradient gels, copolymerised with 0.1% gelatine (Gibco Ltd). After washing to remove the SDS, electrophoresis gels were incubated overnight in 0.1 M Tris buffer, pH 7 at 37°C. Zones of proteolysis were visualised by Coomassie staining (0.1% Coomassie blue R-250 in 25% methanol, 10% acetic acid and 1% glycerol).

Inhibitor sensitivity. The sensitivity to protease inhibitors was tested by determining the hydrolysis of azocasein after preincubation with protease inhibitors. Inhibitors specific for serine proteinases: phenylmethane-sulphonyl-fluoride (PMSF), 1 mM and tosyl lysyl chloromethyl ketone (TLCK), 1 mM; cysteine proteinases: L-trans-epoxysuccinyl-leucyl-amide-4-guanidino-butane (E64), 50 µm and N-ethyl-maleimide (NEM), 1 mM; metalloproteinases: ortho-phenanthroline (1,10 Phe), 2 mM and ethylenediaminetetra-acetic acid (EDTA), 2 mM; aspartyl proteinases: pepstatin, 2 µM and the thiol protecting agent DTT, 2 mM were used. Routinely, 20 µl of sample were preincubated for 1 hour at room temperature with 3 µl of inhibitor in 100 µl buffer, to give the above concentrations of the inhibitors and the enzyme activities were determined as described above with azocasein as substrate. All inhibitors were purchased from Sigma Chemicals Co.

Anti-*T. spiralis* antiserum. Mouse anti-*T. spiralis* antisera were prepared from mice infected 3 times with 150 larvae each. Sera were collected 21 days after the final infection. IgG was purified from sera by affinity chromatography using Protein G-Sepharose 4B (Sigma Chemicals Co) according to the manufacturer's instructions.

Inhibition of proteinases activity by antibody. The inhibition of protease activity by IgG was measured spectrophotometrically using azocasein as described elsewhere (Todorova et al. 1995). Briefly, the ES samples were preincubated with up to 100 µg IgG isolated from sera of normal animals and animals infected with *T. spiralis* for 1 hour at 37°C and the effect on the azocasein degradation was measured as described above.

Western blotting. For Western blot analysis ES were electrophoresed in a 10% SDS-PAGE and electroblotted overnight at 4°C onto nitrocellulose in Tris-glycine buffer, containing 0.1% SDS using a Mini-Trans-blot transfer cell (Bio-Rad). The blots were blocked for 1 hour in 5% skimmed milk in 0.1% Tween in Tris-buffered saline. The mouse anti-*T. spiralis* IgG was added at 1:100 dilution in the buffer described above for 90 min. After washing (three times) the blots were incubated with rabbit anti-mouse alkaline phosphatase-conjugated IgG (1:1000), washed again and the reaction was developed with NBT/BCIP [66 µl NBT (0.3 mg in 1 ml of 70% DMF) and 33 µl BCIP (0.15 mg in 1 ml of 100% DMF) up to 10 ml with alkaline phosphatase buffer (100 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris, pH 9.5)].

RESULTS

Proteinases pH optimum and molecular sizes. The proteolytic enzymes in the larval ES were found to degrade azocasein over a broad pH range (Fig. 1). Peak activity occurred at pH 7, with high activities also at pH 5 and pH 6.

Gelatin-substrate gel analysis showed zones of proteolysis at approximately 25 (a doublet), 35, 40, and 55 kDa (Fig. 2, lane 1). The proteolytic activity of larval ES was compared with that of adult ES (Fig. 2, lane 3). Adult parasites showed proteolysis at approximately 18, 25 (a doublet), 38, 40, and 42 kDa. Proteinases with approximate molecular sizes of 25 and 40 kDa were present in the ES of both larvae and adult parasites, while enzymes with molecular sizes of approximately 35 and 55 kDa for larvae, and 18, 38 and 42 kDa for adults, were stage-specific. In the present study we have used 5-25% gradient gels instead of homogeneous 10% gels we used before (Todorova et al. 1995) and obtained a better resolution of the proteolytic profile in the zone of 15-60 kDa. Here we found several discrete proteinases of adult ES in the zone of 25–42 kDa which were not detected in the homogeneous gels.

Table 1. Proteolytic activity of ES of *Trichinella spiralis* larvae (L1). Data are expressed as change in absorbance (absorbance units × 10^{-3}/16 h.). Mean values of three observations are presented.

Type of enzyme activity indicating	Substrate	pH 5	pH 6	pH 7
General	Azocasein	52.3	88.2	137.3
Collagenase	Azocoll	183.2	108.7	65.3
Elastase	Elastin-orcein	95.1	128.2	172.1

Table 2. The effect of protease inhibitors on proteolytic activity of *Trichinella spiralis* larval ES assessed in azocasein degradation assay. The inhibition of proteolytic activity is expressed as a reduction in absorbance compared to an inhibitor-free control. The means of two observations are shown.

Inhibitor	Inhibition %
PMSF	62
TLCK	37
E64	12
NEM	0
1,10 Phe	15
EDTA	7
pepstatin	0
DTT	0

Substrate specificity. The proteolytic activities of larval ES were examined at pH 5, 6, and 7 using three chromogenic substrates: azocasein, azocoll and elastin-orcein (Table 1). The results indicated that larval ES contained collagenolytic and elastolytic activities. The collagenolytic activity was maximal at pH 5, while elastolytic had a maximal value at pH 7. The activity against the general proteinase substrate azocasein was maximal at pH 7.

Inhibitor sensitivity of ES proteinases. The effects of various inhibitors on the proteolytic activity of larval ES against azocasein as a substrate are shown in Table 2. ES proteinases were markedly inhibited by serine-specific inhibitors PMSF (62%) and TLCK (37%). Inhibition was established also with E64 (12%) and 1,10 Phe (15%), indicating the presence of cysteine and metalloproteinases. The predominance of serine type of proteinases was confirmed by the substrate gel electrophoresis. After preincubation with the serine specific inhibitor PMSF the protease activities of larval ES in the zone between 25 and 40 kDa were completely abolished (Fig. 2, lane 2). The effect of PMSF on the proteolytic activity of larval ES was compared with that on the adult parasite ES. Most of the proteinase activities in the ES of adult parasites were also totally or partially inhibited. (Fig. 2, lane 4). Proteolysis at approximately 25 kDa was totally inhibited and others were reduced.

Antibody inhibition of ES proteinases. The effect of IgG from *T. spiralis*-infected and control (uninfected) animals on the proteolytic activity of larval ES was assessed in azocasein degradation assay. The results are presented in Fig. 3. Preincubation of ES with up to 100 μg IgG from *T. spiralis*-infected animals showed a 63% reduction of azocasein-proteolysis compared with about 10% reduction with normal IgG. Gelatin-substrate gels confirmed the antibody-mediated inhibition of proteolytic activity of larval ES, although there was considerable variation between gels (not shown).

Antibody recognition of ES components. Using Western blot analysis we found that a component of larval ES with molecular size of approximately 35 kDa was recognised by IgG isolated from antisera of mice infected with *T. spiralis* (Fig. 4).

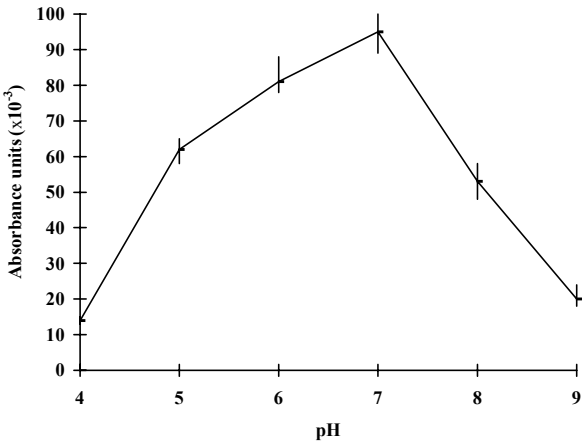

Fig. 1. The effect of pH on azocasein degradation by proteinases in larval ES of *Trichinella spiralis*. The means of three observations are shown, with error bars representing the S.D.

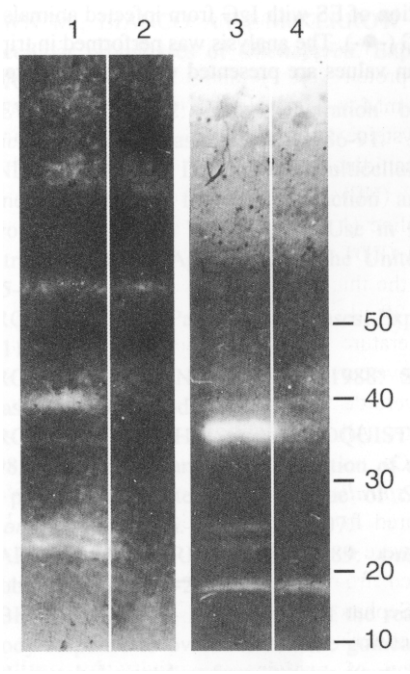

Fig. 2. Proteolytic activities of larval (lane 1) and adult (lane 3) *Trichinella spiralis* ES. Preincubation of ES with serine protease inhibitor PMSF prior to electrophoresis (larval ES, lane 2; adult ES, lane 4) showed that the proteolysis is totally or partially inhibited.

DISCUSSION

Proteolytic enzymes secreted by parasites are thought to play a key role in the processes of penetration and migration through the host tissues. It has been stated that all of the proteinases secreted by tissue-invading parasites fall into two of the classes of proteinases – serine and metallo (McKerrow 1989). Proteinases of these classes have been demonstrated in studies on the secreted products of *Anisakis simplex* (Sakanari and McKerrow 1989), *Onchocerca lienalis*, *O. cervicalis* and *O. cervipedis* (Lackey et al. 1989), *Schistosoma mansoni* (McKerrow et al. 1985), *Ancylostoma caninum* (Hotez et al. 1985), *Dictyocaulus viviparus* (Britton et al. 1992) and adult *Trichinella spiralis* (Todorova et al. 1995). Criado-Fornelio et al. (1992) established the

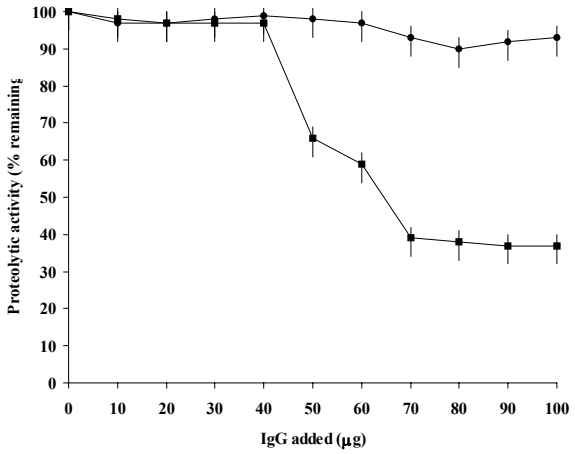

Fig. 3. The effect of IgG isolated from infected and uninfected mouse sera on the proteolytic activity of larval *Trichinella spiralis* ES assessed in azocasein degradation assay. Preincubation of ES with IgG from infected animals (-■-) and normal IgG (-●-). The analysis was performed in triplicate and of the mean values are presented with error bars representing the S.D.

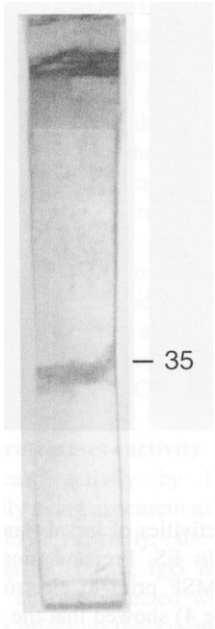

Fig. 4. Western blot analysis of larval ES of *Trichinella spiralis*. Following SDS-PAGE of the ES the blots were probed with IgG isolated from sera of *T. spiralis*-infected and normal mice. No reaction was found with IgG isolated from normal mouse sera (not shown).

presence of proteolytic activities in the ES of *T. spiralis* larvae, but the molecular sizes, pH optimum, and type of the proteinases have not been defined. In this study, the proteolytic activity of the *in vitro* released ES of larval stage (L1) of *T. spiralis* were examined according to their pH optimum, activity against some protein substrates and inhibitor sensitivity. The inhibitor studies showed that serine proteinases predominated. ES proteinases degraded azocasein, azocoll and elastin-orcein over a broad pH range with peak activities at pH 5 for the degradation of azocoll and pH 7 for azocasein and elastin-orcein. These results would argue for the presence of collagenolytic and elastinolytic activities of ES of *T. spiralis* larvae. With respect to the possible function of secreted parasitic proteinases *in vivo*, it has been proposed that collagenolytic and elastolytic activities are consistent with tissue migration (Matthews 1982, McKerrow et al. 1988, Chavez-Olortegui et al. 1992). These proteinases might also be active in the processes of parasite transformation and counter-immunity.

The present study established that antibody stimulated by infection can inhibit the proteolytic activity of ES. Antibody-mediated inhibition of parasite proteinases has been reported in investigations on experimental infection with *Schistosoma mansoni* (Auriault et al. 1981), *Ascaris suum* (Knox and Kennedy 1988), *Dictyocaulus viviparus* (Britton et al. 1992), *Haemonchus contortus* (Knox et al. 1993), *Trichinella spiralis* (Armas-Serra et al. 1995a, Todorova et al. 1995) and it is possible that the antibody response can minimise the direct damage of host tissues by inhibition of proteinase activity.

A component of larval ES was recognised by IgG isolated from anti-*Trichinella spiralis* serum as shown by Western blot analysis. The presence of a proteinase with the same molecular size was found by means of substrate SDS-PAGE and it could be the same, which was recognised by the immune antibody. Moreover, a 35 kDa proteinase with properties of both serine and cysteine proteinases has been purified from ES of *T. spiralis* muscle larvae (Armas-Serra et al. 1995a, b) and this has been shown to have antigenic properties. The recognition of this *in vitro* secreted suspected serine protease by IgG isolated from antisera of mice infected with *T. spiralis* indicates that it is a target of the immune response to infection with *T. spiralis* and suggests that it is secreted also *in vivo*.

In summary, this study showed that the infective larvae of *T. spiralis* secrete *in vitro* a number of proteinases predominantly of the serine type. These enzymes showed azocollytic and elastolytic activities, probably implicated in tissue penetration. They are targets of inhibitory host antibody responses in *vivo*, which might presumably impair parasite survival. A serine protease with molecular size of approximately

35 kDa is recognised by immune antibody and is specific for the larval stage of the parasite. The information provided could be valuable in further investigations, including purification techniques, design of gene cloning strategies and development of disease-stage markers or diagnostic reagents.

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