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# Partial characterization of serine proteinases secreted by adult *Trichinella spiralis*

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**Abstract** Serine proteinases secreted by adult *Trichinella spiralis* were isolated from excretory/secretory products (ES) of in-vitro-cultured parasites by affinity chromatography with *p*-benzamidine-celite. The purified enzymes had molecular weights of approximately 18, 40, and 50 kDa and displayed enzyme activity against a range of low-molecular-weight substrates, gelatin, and azocasein. The antigenicity of these parasite proteinases was demonstrated by the inhibition of enzymatic activity with IgG purified from infected hosts. The inactivation of major secreted proteinases of adult *T. spiralis* by immune antibody could presumably contribute to impairment of the survival of the parasite in sensitized hosts.

## Introduction

Members of the serine class of proteinases are widely distributed in nature and are found through prokaryotic and eukaryotic organisms. In parasitic infections they have been implicated in such diverse processes as facilitation of the invasion of host tissues, digestion of host proteins, and evasion of host immune responses (Marikowsky et al. 1988; McKerrow 1989; Monroy et al. 1989; Fishelson et al. 1992). Serine proteinases, including secreted enzymes, have been established in studies of *Schistosoma mansoni* (McKerrow et al. 1985; Newport et al. 1988), *Onchocerca lienalis* (Lackey et al. 1989), *Toxocara canis* (Robertson et al. 1989), and *Trichinella spiralis* (Armas-Serra et al. 1995; Todorova et al. 1995). In a series of reports the use of parasite proteinases as a source of diagnostic reagents has been proposed (McKerrow 1989; Song and Chappell 1993; Knox 1994), and an essential stage in this process is the acquisition of information about their biochemical characteristics and antigenicity.

*T. spiralis* is a parasitic nematode of a wide variety of mammals, including humans. The complete life cycle of the parasite occurs in a single host and comprises the adult stage in the host gastrointestinal tract, a migratory phase during which the newborn larvae pass through the bloodstream and lymphatics to the skeletal muscle cells, and encapsulated larvae in the muscles (Despommier 1983). Adult *T. spiralis* secrete "in vitro" a variety of proteinases, which degrade fibrinogen and plasminogen and are inhibited by antibody from mice rendered immune to *T. spiralis* (Todorova et al. 1995).

The present report describes the preliminary characterization of serine proteinases isolated from excretory/secretory products (ES) of adult *T. spiralis*. These enzymes were defined according to their molecular size and substrate specificity. It was also important that we determine whether they were antigenic in the context of a full-course infection and what effect the immune response might have on their activity. In addition, we examined whether the secreted serine proteinases represented allergens.

## Materials and methods

### Parasites and preparation of ES

ES of adult *Trichinella spiralis* were obtained as described elsewhere (Todorova et al. 1995). The protein concentration of ES typically ranged between 50 and 100 μg/ml.

### Substrate sodium dodecyl sulfate-polyacrylamide gel electrophoresis

ES were separated under nonreducing conditions without boiling of the samples using 5–25% polyacrylamide gradient gels copolymerized with 0.1% gelatin (Gibco Ltd.). The procedure has been described in detail elsewhere (Todorova et al. 1995).

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### Inhibitor sensitivity of ES proteinases

The effect on protease activity of inhibitors with specificity for serine (phenylmethanesulfonyl fluoride, PMSF, 1 m*M*), cysteine (L-transepoxy-succinyl-4-guanidinoleucylamidobutane, E64, 20 μ*M*), metalloproteinases (1,10-phenantroline, 1,10-Phe, 2 m*M*), and aspartyl proteinases (pepstatin, 10 μ*M*) was assessed as described by Todorova et al. (1995).

### Affinity chromatography with benzamidine-celite

A batch affinity-purification technique using *p*-Aminobenzamidine-Celite (Sigma Ltd.) was applied for the purification of serine proteinases from ES of adult parasites cultivated in vitro. Pooled secretions with a protein concentration of 90 μg/ml as estimated using a Coomassie-blue-based assay (Pierce Chemical Co., Illinois) were subjected to affinity chromatography as follows: 500 μl ES was added to 10 mg benzamidine-celite in 1 ml phosphate buffer (0.1 *M* $Na_2HPO_4$ and 0.4 *M* NaCl, pH 8.0), and the solution was incubated for 30 min at 4 °C. The mixture was centrifuged for 2 min at 5000 rpm, and to the pellet was added 1 ml elution buffer (0.1 *M* Na-acetate and 0.4 *M* NaCl). After 2 min of incubation at 4 °C the mixture was centrifuged at 5000 rpm and the supernatant was dialyzed against phosphate-buffered saline (PBS) and concentrated. The purification was monitored using sodium dodecyl sulfate (SDS)-polyacrylamide gels copolymerized with gelatin after preincubation with PMSF.

### Enzyme activity

Proteolytic activity was measured spectrophotometrically using a chromogenic substrate, azocasein (Todorova et al. 1995). Low-molecular-weight substrate solutions, usually at concentrations of 1 mg/ml, were prepared in dimethylsulfoxide (DMSO). The following substrates (purchased from Sigma Ltd.) were used: *N*-carbobenxoxy-*l*-tyrosine-4-nitrophenol ester (Cbz-L-Tyr-NPE), Cbz-L-Phe-4-NPE, Cbz-L-Lys-4-NPE, Cbz-L-Arg-4-NPE, Cbz-L-Ala-4-NPE, *N*-benzoyl-L-tyrosine-*p*-nitroanilide (Bz-L-Tyr-NA), and Bz-Arg-NA. Reaction mixtures comprised 20 μl sample, 100 μl buffer (0.1 *M* Tris, pH 7.5), 50 μl DMSO, and 5 μl substrate solution. Initial (time zero) and final (30 min) absorbance values were measured at 405 nm, over which period the increase in absorbance was linear with time. All activities were corrected for nonenzymatic hydrolysis by subtraction of the appropriate reagent blank (Knox and Kennedy 1988).

### Antisera and purification of IgG

Mouse anti-*T. spiralis* sera were prepared in mice as described elsewhere (Todorova et al. 1995).

### Inhibition of protease activity by antibody

The inhibition of protease activity by IgG was determined as described previously (Todorova et al. 1995).

### Passive cutaneous anaphylaxis

Two inbred Wistar rats were injected intradermally with 100-μl samples of anti-*T. spiralis* serum, normal rat serum, and PBS. After 24 h the rats were given an i.v. injection of 50 μg Ag (benzamidine-purified serine proteinases) with 5 mg Evans blue in PBS, and the diameters of the reaction spots were measured 30 min later.

## Results

### Inhibitor sensitivity of ES proteinases

Samples of ES released by adult *Trichinella spiralis* were subjected to separation in matrices containing gelatin as a general substrate of proteolytic enzymes (Fig. 1). We detected multiple bands of protease activity at between 18 and 50 kDa (Fig. 1, lane 5). The effect of class-differentiating protease inhibitors on ES is presented in Fig. 1 (lanes 1–4). ES of adult parasites predominantly contained serine proteinases (lane 1), but the additional presence of metalloproteinases (lane 2) and cysteine proteinases (lane 3) was established. There were protein bands at approximately 18, 25 (a doublet), 38, 40, 42, and 50 kDa. Better resolution of the proteolytic profile in the zone of 20–60 kDa was obtained using gradient gels as compared with the homogeneous 10% gels used in previous studies (Todorova et al. 1995).

### Affinity purification of serine proteinases

The results obtained using affinity chromatography are presented in Fig. 2. The proteinases that were eluted from the benzamidine-celite had approximate molecular weights of 18, 40, and 50 kDa as shown by substrate SDS-PAGE (Fig. 2, lane 3). These corresponded to proteolytic activities established in the total ES of adult *T. spiralis* (Fig. 2, lane 1). Preincubation of benzamidine-purified serine proteinases with PMSF showed total inactivation of their enzyme activity (Fig. 2, lane 4).

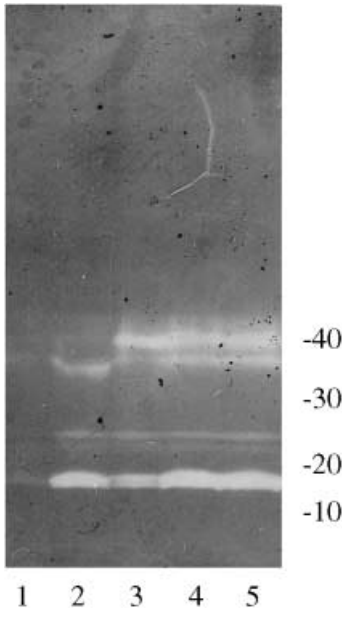


**Fig. 1** In-vitro-secreted proteinases of adult *Trichinella spiralis* and the effect of a panel of class-differentiating proteinase inhibitors on proteolytic activities (*Lane 1* ES + PMSF, *lane 2* ES + 1,10-Phe, *lane 3* ES + E64, *lane 4* pepstatin, *lane 5* ES control)

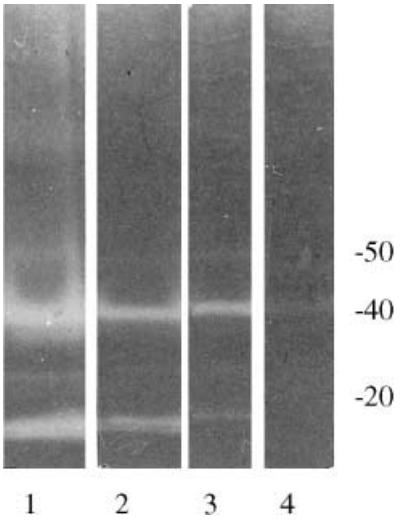


**Fig. 2** Serine proteinases purified from ES of adult *T. spiralis* by affinity chromatography using benzamidine-celite (*Lane 1* ES, *lane 2* ES + PMSF, *lane 3* benzamidine-purified serine proteinases, *lane 4* benzamidine-purified serine proteinases + PMSF)

### Substrate specificity

The substrate specificity was examined at pH 7.5, which was the optimal pH for proteolytic degradation established for ES of adult *T. spiralis* (Todorova et al. 1995). A range of low-molecular-weight substrates specific for chymotrypsin, trypsin, and elastase were used (Table 1). The results indicated that the enzyme activity of secreted serine proteinases purified by benzamidine-celite was mainly chymotryptic and elastolytic.

### Inhibition by immune IgG and allergenicity

Preincubation of benzamidine-purified serine proteinases with 100 μg IgG isolated from sera of immune mice led to a 90% loss of activity in comparison with that observed in a control containing serine proteinases alone. IgG isolated from normal mice reduced the proteolytic activity by 10%.

**Table 1** Proteolytic activity of serine proteinases purified by affinity chromatography against various substrates[a]

| Type of enzyme activity | Substrate | Proteolytic activity |
|---|---|---|
| Chymotryptic (esterolytic) | Cbz-L-Tyr-4-NPE | 237 |
| | Cbz-L-Phe-4-NPE | 72 |
| Tryptic (esterolytic) | Cbz-L-Lys-4-NPE | 43 |
| | Cbz-L-Arg-4-NPE | 21 |
| Elastolytic | Cbz-L-Ala-4-NPE | 125 |
| Chymotryptic (amidolytic) | Bz-L-Tyr-NA | 48 |
| Tryptic (amidolytic) | Bz-L-Arg-NA | 18 |

[a] Data represent mean values obtained for the change in absorbency (absorbance units $\times 10^{-3}$) per 30 min in assays carried out in triplicate

The results obtained for passive cutaneous anaphylaxis (PCA) showed a positive reaction against benzamidine-purified serine proteinases, which indicated that these enzymes had allergenic properties. No reaction was established with normal serum or PBS.

## Discussion

Using affinity chromatography with benzamidine-celite, we partially purified serine proteinases from the ES products of adult *Trichinella spiralis*. Benzamidine is a reversible inhibitor of serine proteinases (North 1989) that, when coupled to a suitable matrix, can be used for purification of serine proteinases (Holmberg et al. 1976; Mizuno et al. 1987).The purified enzymes showed proteolytic activity against a variety of substrates and were targets of humoral immune responses.

The use of a range of low-molecular-weight substrates enabled differentiation of the enzymatic activity of purified enzymes into chymotrypsin-, elastase-, and – to a lesser degree – trypsin-like proteinase activity. There is no indication of the function of ES in vivo, although the elastase activity found for the serine proteinases would play a role in the degradation of intestinal tissues and, hence, would facilitate either the penetration of the parasites into host enterocytes or the release of nutrients. These proteinases might also be active in the processes of parasite transformation and counterimmunity. Previous results (Todorova et al. 1995) had established that ES of adult *T. spiralis* degraded fibrinogen and plasminogen, which suggests a role in anticoagulation or feeding.

The present finding that antibody stimulated by infection with *T. spiralis* could inhibit serine proteinases raises the question as to whether the secreted serine proteinases might be capable of inducing protective immunity in vivo. Secreted proteinases have been found to be the targets of significant antibody responses in a number of nematodiases (Ogilvie and deSavigny 1982; Kennedy and Qureshi 1986). Moreover, this response is stronger than that elicited against parasite somatic components. Protective immune responses have been elicited by a proteolytic enzyme present in erythrocytes infected with *Babesia bovis* (Commins et al. 1985). It is possible that the antibody response might minimize the direct damage incurred by host tissues as a result of inhibition of proteinase activity. It is also known that ES products of some parasitic nematodes contain allergens and IgE-potentiating factors (Stromberg 1979, 1980; Ogilvie and deSavigny 1982). Kennedy et al. (1991) established that the ES components of adult *T. spiralis* elicited precipitating antibody responses in immune mice. The positive PCA results obtained in the present study indicate the allergenicity of *T. spiralis*-secreted serine proteinases.

The secreted proteinases of *T. spiralis* are undoubtedly important for the parasite, and their inactivation by antibodies may have detrimental effects on the parasite's survival. The antibody-mediated inhibition of serine

proteinases argues for their further purification and for characterization of the individual enzymes such that their role in pathogenesis and immunity can be examined.

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