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ORIGINAL PAPER

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Biochemical comparison of the serine protease (elastase) activities in cercarial secretions from *Trichobilharzia ocellata* and *Schistosoma mansoni*

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Abstract We report on serine protease activity in cercarial secretions (CSs) from the bird parasite *Trichobilharzia ocellata*. Using a colorigenic substrate, the biochemical properties of this enzyme were studied and its activity was compared to the homologous one in CSs from the human parasite *Schistosoma mansoni*. The specific serine protease activity was always 2- to 3-fold higher in CSs from *T. ocellata* compared to *S. mansoni*. The enzyme has its optimal activity at pH 10.5, is Ca^{2+}-dependent (inhibition with EDTA) and has a trypsin-like (inhibition with anti-pain) serine proteinase activity (inhibition with PMSF and aprotinin). The K_m value of the serine protease from *T. ocellata* was higher than that of *S. mansoni*, and the K_i values for several inhibitors were generally lower for the enzyme of *T. ocellata* than that of *S. mansoni* except for EDTA. The enzyme activities from both parasites had a molecular weight of 30 kDa in gelatin-SDS-polyacrylamide gels. The intensity of the gelatin digestion bands was stronger with the *T. ocellata* than with the *S. mansoni* enzyme.

Introduction

Cercariae of the parasite genus *Trichobilharzia* emerge from their snail intermediate host (*Lymnaea stagnalis*) to infect water birds which are the definitive hosts. The penetration of *Trichobilharzia* cercariae includes the action of penetration glands and seems to be similar to the human parasite *Schistosoma mansoni* (Bourns et al. 1973). Cercariae of *Trichobilharzia* may also accidentally invade humans and then stimulate a pronounced skin inflammatory reaction (cercarial dermatitis). Although the parasites do not establish themselves in mammals and, therefore, do not cause a transmissible infection, it is important not to underestimate mammalian infections by bird schistosomes. Thus, migrating bird schistosomes can cause not only pulmonary hemorrhages (Oliver 1953) but also neural disorders and paralysis in laboratory mice (Horak et al. 1999).

Cercariae penetrate the skin of their host with the help of secretions from the acetabular glands (McKerrow et al. 1991). These cercarial secretions (CSs) contain proteases, the release of which is stimulated by fatty acids normally found on human skin (Shiff et al. 1972). One of the predominant proteins in the CSs is cercarial elastase (CE). This enzyme has been purified and characterized from *S. mansoni* (McKerrow et al. 1985). It has an apparent molecular weight of 28 kDa (Ghendler et al. 1996) to 30 kDa (McKerrow et al. 1985), an isoelectric point of 8 and shows optimal activity at pH 9 (McKerrow et al. 1985). The CE is synthesized during the sporocyst stage, stored in the gland cells and plays a vital role in aiding host invasion by dissolving the host's skin tissues (McKerrow and Doenhoff 1988).

Following infection, the CSs contain the first potentially immunogenic material that may stimulate the host immune response. The CE from *S. mansoni* was reported by Toy et al. (1987) to stimulate transient IgM antibodies against this enzyme in mice by 1 week after infection. In the same report, CE was also strongly recognized by sera from humans infected with *S. mansoni*, *Schistosoma japonicum* or *Schistosoma haematobium*. However, the reactivity with sera from swimmer's itch (exposed to *Trichobilharzia*) was very poor (Toy et al. 1987). In contrast, we did not observe an apparent antibody response against CE in animals or humans following natural infection, although experimental immunization with purified CE did induce antibody production (Bahgat et al. 2001). This immunological discrepancy with respect to the reactivity of patients'

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sera still needs clarification. Rather, other cercarial antigens appear to be responsible for the reactions of CSs with the sera of schistosomiasis patients as observed by Kolarova et al. (1994) and by ourselves (Bahgat et al. 2001).

Whereas the CE from *S. mansoni* has been the subject of several biochemical studies (see above), a homologous or analogous enzymatic activity has not been characterized from CSs of *Trichobilharzia ocellata*. Since all schistosomes infect their hosts by skin penetration, we suspected that *T. ocellata* may use similar cercarial enzymes to invade its host. We have recently shown that CSs from several species of *Schistosoma* and from *T. ocellata* have a proteolytically active enzyme, CE, which occurs around 30 kDa in gelatin gels. We also showed cross-reactivity between anti-sera raised against gel-purified CE from *S. mansoni* with the cercarial glands of *T. ocellata* (Bahgat et al. 2001). Here, we compare this serine protease activity from cercariae of *T. ocellata* with that of cercariae of *S. mansoni*, at the biochemical level.

Materials and methods

Parasites

The life cycle of *S. mansoni* (strain from Puerto Rico) was maintained in *Biomphalaria glabrata* and NMRI mice as previously described (Ruppel et al. 1990). *L. stagnalis* snails infected with *T. ocellata* were kindly provided by W. Haas (University of Erlangen).

Preparation of the CSs from different schistosome species

Infected *B. glabrata* and *L. stagnalis* were placed in beakers with deionized water. After 15 min the water containing the snails' faeces was replaced by fresh water and the snails were exposed to light for 1 h. The cercariae-containing water (500–1,000 cercariae/ml) was slowly decanted into a new beaker. A total of 10,000 actively swimming cercariae was then poured onto a Petri dish which had previously been painted with linoleic acid (0.9 g/ml; Sigma, St. Louis, Mo., USA) and air dried. The plates were kept at 37°C for 30 min. After this incubation the water containing secretions and dead cercariae was collected in 15 ml Falcon tubes and the dead cercariae were sedimented on ice. The water containing the CSs was collected and spun down at 3,000 *g* for 2 min to ensure that the secretions were completely free from cercarial debris. The protein concentration of the CSs was measured using the BCA protein determination kit (Pierce, Ill., USA).

Assay for proteinase activity

Protease activities in CSs from *S. mansoni* and *T. ocellata* were assessed with the substrate L-1195 (Boc-Val-Leu-Gly-Arg-PNA; Bachem Biochemica, Heidelberg, Germany). L-1195 reacts specifically with trypsin-like serine proteinases (Iwanaga et al. 1978). The enzymatic hydrolysis of the covalent bond between the arginine and the *p*-nitroanilide group results in the release of yellow-colored *p*-nitroaniline which was recorded by measuring the absorption at 405 nm. A stock solution of the substrate (10 mg/ml) was prepared in dimethylsulfoxide (DMSO) and then diluted to the desired final concentration with the substrate buffer (30 mM TRIS-HCl, 60 mM NaCl, 0.05% NaN_3, pH 10.5). Specific protease activity is expressed in nM *p*-nitroaniline/min per µg protein. For data presented in Fig. 1A, additional pH values were used to determine the optimum pH. All incubations were done for 6 h at 37°C.

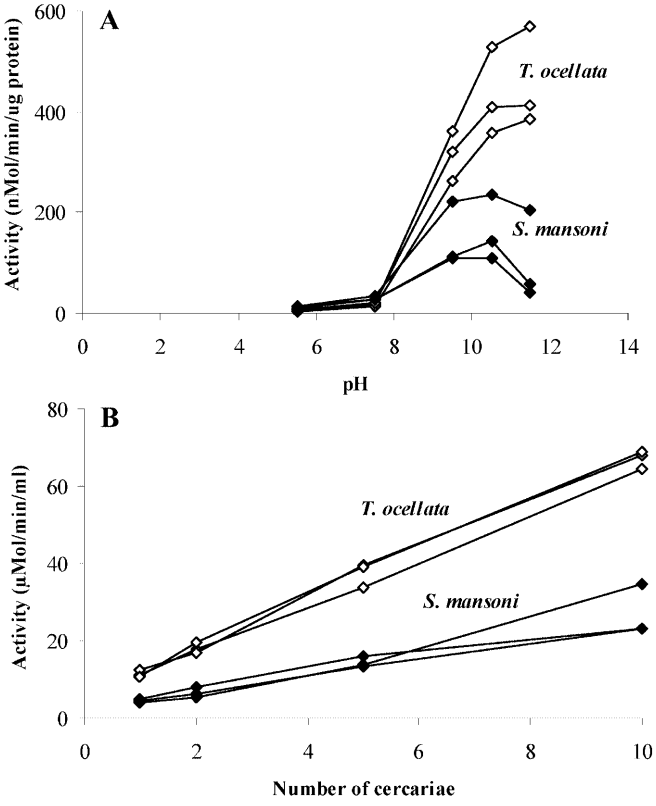

Fig. 1A, B Protease activity in cercarial secretions. Activity was measured by hydrolysis of the substrate L-1195 (150 µg/ml). **A** pH dependency of the activity as determined at a constant protein concentration of secretions (20 µg/ml). **B** Dependency on cercarial numbers. The activity was tested in three different preparations from *Trichobilharzia ocellata* and *Schistosoma mansoni*. Each value is the mean of duplicate measurements

Inhibition assay for enzyme activity

Inhibition of protease activity was studied with the following protease inhibitors: ethylenediamine-tetraacetic acid (EDTA, disodium salt; Roth, Karlsruhe, Germany), phenylmethylsulfonylflouride (PMSF; Sigma), anti-pain and aprotinin (both from Boehringer Mannheim, Germany). Stock solutions of the inhibitors were prepared in water except for PMSF which was dissolved in 70% ethanol. For performing inhibition assays, 50 µl CSs were first incubated for 10 min with 10 µl of the inhibitor appropriately diluted to give the desired final concentration. Control assays contained 50 µl CSs, 10 µl of either water or 70% ethanol and the substrate and were carried out as described above.

Enzyme kinetics

For calculating the Michaelis-Menten constants (K_m), maximum velocity (V_{max}) and inhibitory coefficients (K_i), serial dilutions of the substrate L-1195 were prepared ranging from 0.5 mM to 0.015 mM. In addition, serial dilutions of the inhibitors were prepared. For testing the inhibition kinetics of the enzyme activity the final inhibitor concentrations were: PMSF at 0.5, 0.25 and 0.125 mM for both parasites; EDTA at 1.0, 0.5, 0.125 mM for *T. ocellata* and 0.5, 0.25 and 0.125 mM for *S. mansoni*; anti-pain at 2.0, 1.0 and 0.5 µM and aprotinin at 2.0, 1.0 and 0.5 nM for both

species. CSs (50 µl; 20 µg protein/ml) were mixed with the desired inhibitor (10 µl at the appropriate concentration), incubated for 10 min at room temperature and then mixed with the serially diluted substrate. Control assays contained water (10 µl; instead of EDTA, aprotinin or anti-pain) or 70% ethanol (10 µl; instead of PMSF) and were carried out as described above.

Electrophoresis of CSs

The protein patterns of CSs from *S. mansoni* and *T. ocellata* were analyzed by sodium dodecylsulfate-polyacrylamide gel electrophoresis (Laemmli 1970) through 4% stacking and 10% resolving gels (55×85×1 mm) in a Mini-Protean II electrophoresis chamber (Bio-Rad Laboratories). Low range molecular weight (MW) marker proteins ranging from 21 to 97 kDa (Bio-Rad Laboratories, Munich, Germany) were included on the same gel. Gels were silver-stained according to the method of Morrissey (1981).

SDS-gelatin gels for detecting proteolytic activity

CE activity in the CSs was characterized with respect to MW using SDS-polyacrylamide gels copolymerized with gelatin (Heussen and Dowdle 1980). These gels differ from regular SDS-polyacrylamide gels in two aspects. First, a proteinase substrate (in our case 6% gelatin) is added before initiating the polymerization of acrylamide. Second, the sample buffer does not contain reducing agents and the sample is not boiled. After electrophoresis, proteins were allowed to renature by removing the SDS. This was accomplished by incubating the gel in 2.5% Triton-X-100 in water with gentle shaking for 30 min and with one change at room temperature. The gel was then incubated overnight at 37°C with gentle shaking in 30 mM TRIS-HCl, pH 9.5, containing 60 mM NaCl and 0.05% NaN_3, subsequently stained with 0.5% Coomassie blue (in 10% acetic acid, 5% methanol) and destained. Proteolytic activity was evidenced as unstained bands in the blue gel.

Results and discussion

Protease activity in CSs was tested by hydrolysis of L-1195 as measured by OD at 405 nm. The pH dependency of this activity was studied for both *T. ocellata* and *S. mansoni* and results are shown in Fig. 1A. The enzyme activity of *S. mansoni* had an alkaline optimum pH value around 10.5. Similarly, McKerrow et al. (1985) reported an optimum pH value of 9 for the elastase from *S. mansoni* cercariae. Since these authors used radiolabeled elastine as substrate, the difference in the pH optima observed by them and by ourselves could be due to the different substrates used. For *T. ocellata*, we observed increasing activity up to pH 11.5 (Fig. 1A). However, since the substrate L-1195 undergoes alkaline hydrolysis above pH 11.5, we could not ascertain that the enzyme of *T. ocellata* shows maximum activity with L-1195 at this pH.

In three independent preparations of CSs from both species that were adjusted to the same protein concentration, the enzyme activity was always 2- to 3-fold higher with *T. ocellata* than with *S. mansoni*. This observation was made at various pH values using the same protein concentration of CSs (Fig 1A) or at pH 10.5 with different numbers of cercariae (Fig. 1B). The higher activity observed with *T. ocellata* could be due to the higher affinity of the enzyme from this species to the substrate used or to a higher concentration of the enzyme in the CSs from *T. ocellata* compared to *S. mansoni*. The difference could also be related to a higher expression of the protease gene at the transcriptional or the translational level.

Inhibition assays for the protease activity in CSs from both species were carried out using serial dilutions of several inhibitors: aprotinin, which inhibits plasmin, kallikrein, trypsin and chymotrypsin activity and which is a known serine protease inhibitor; anti-pain, which inhibits papain and trypsin; EDTA, which inhibits Ca^{2+}-dependent proteases; and PMSF, which is a selective inhibitor for serine proteases. The inhibitors differed greatly in their effects. The enzyme activity was reduced by 90% with 1 mM PMSF or 3 mM EDTA for both species. With anti-pain more than 90% of the enzymatic activity was inhibited at 0.125 µM for

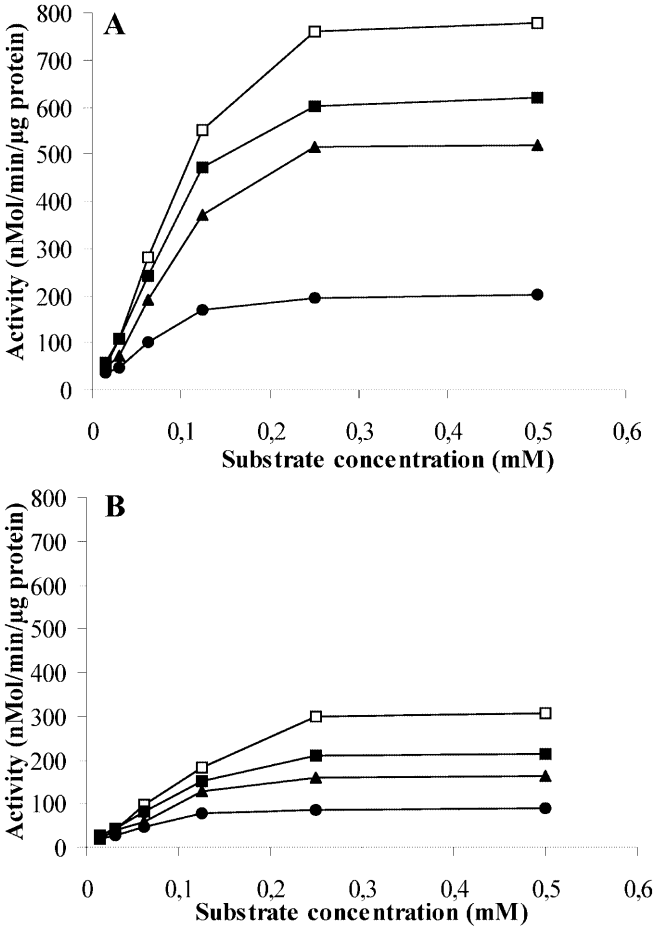

Fig. 2 Calculation of Michaelis-Menten constants (K_m), maximum velocity (V_{max}) for, and inhibition by PMSF of, the protease activity from: **A** *Trichobilharzia ocellata* and **B** *Schistosoma mansoni*. Activity was measured by hydrolysis of L-1195 at the indicated concentrations (*open squares*) The inhibitor PMSF was tested at final concentrations of 0.125 mM (*closed squares*), 0.25 (*triangles*) and 0.5 (*circles*). Cercarial secretions (50 µl; 20 µg protein/ml) were mixed with the inhibitor (10 µl at appropriate concentration), incubated for 10 min at room temperature and then mixed with the serially diluted substrate

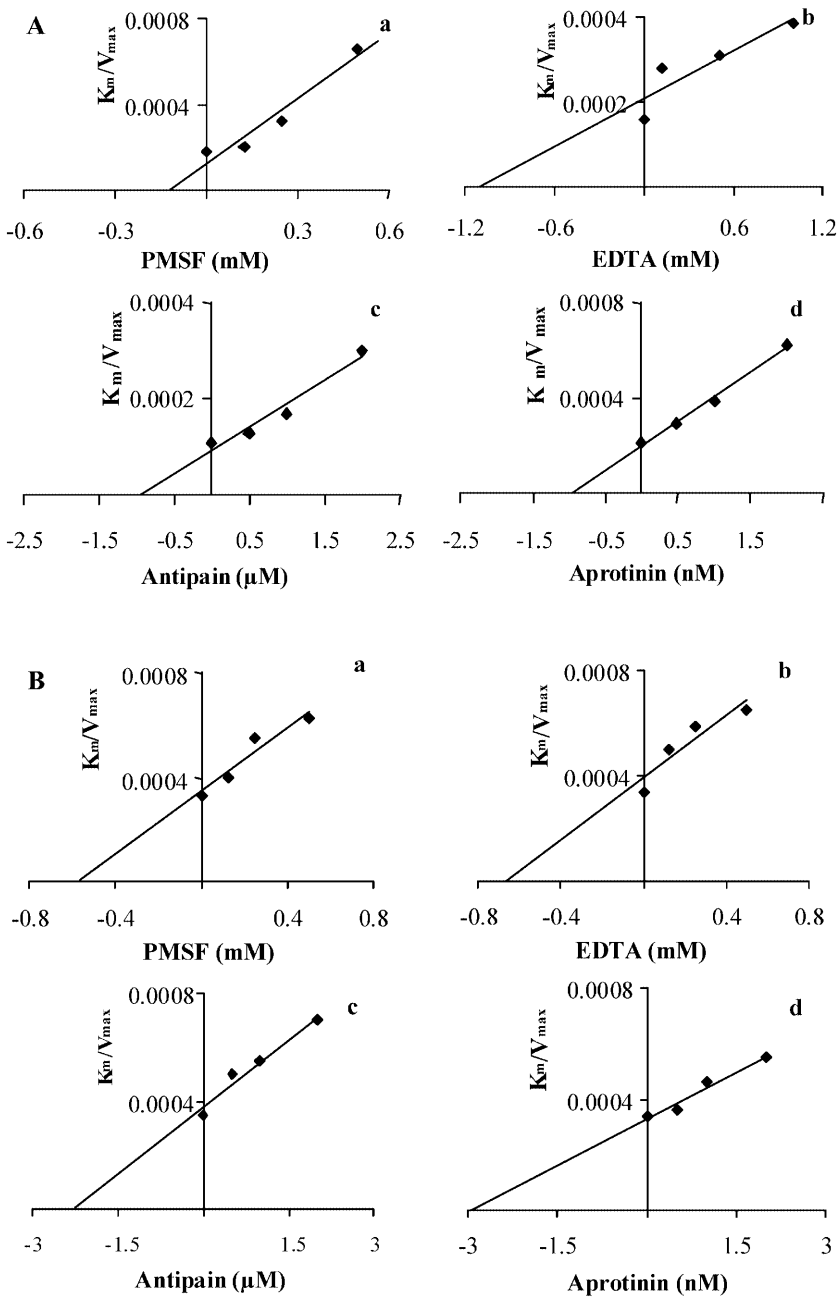

Fig. 3 Dixon plots for different serine protease inhibitors and protease activity from secretions of cercariae from: **A** *Trichobilharzia ocellata* and **B** *Schistosoma mansoni*. The ratio K_m/V_{max} is plotted against the concentrations of different inhibitors, and the K_i values were graphically determined as the intercept with the *abscissa*

T. ocellata CSs, but 0.5 μM was required for *S. mansoni* CSs. In contrast to the other inhibitors, a minute concentration (8 nM) of aprotinin was sufficient to suppress about 90% of the enzyme activity in CSs of both *T. ocellata* and *S. mansoni*. The preliminary inhibition data are not illustrated.

The kinetics of the serine protease activities in CSs of *S. mansoni* and *T. ocellata* were measured with respect to V_{max}, K_m and K_i. The V_{max}- and K_m-values were determined in four independent experiments by plotting the enzymatic activity (nM/min/μg protein) against the substrate concentration (mM) (Fig. 2). The K_m value was 0.095 mM for *S. mansoni* and 0.12 mM for *T. ocellata*. The same experiments were done, again in quadruplicate, in the presence of each of the serially diluted inhibitors. The results obtained with PMSF are included in Fig. 2 and are representative for experiments with

Table 1 K_i values of various protease inhibitors for serine protease activity in secretions of cercariae from *Schistosoma mansoni* and *Trichobilharzia ocellata*

Inhibitor	K_i values for protease of	
	Schistosoma mansoni	*Trichobilharzia ocellata*
PMSF	0.57 mM	0.12 mM
EDTA	0.65 mM	1.10 mM
Anti-pain	2.30 μM	1.00 μM
Aprotinin	3.00 nM	1.00 nM

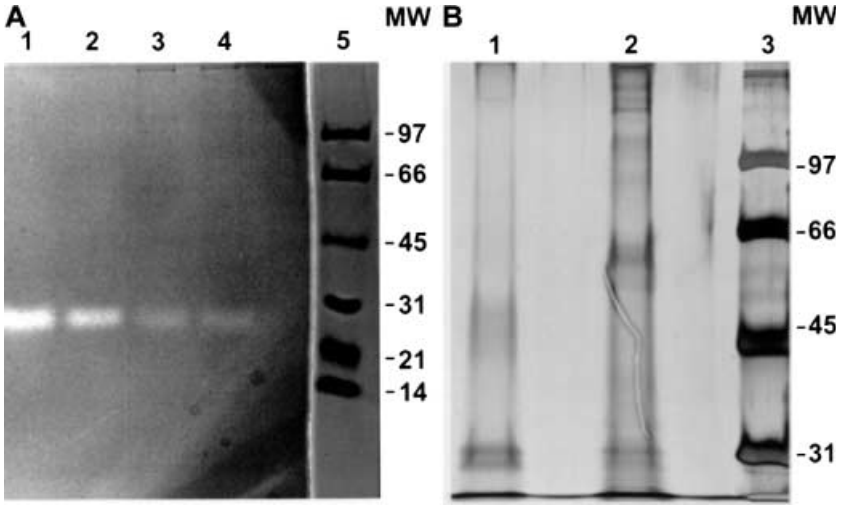

Fig. 4 Serine protease from cercarial secretions in SDS-polyacrylamide gels. Secretions were obtained from cercariae of *Trichobilharzia ocellata* or *Schistosoma mansoni* and separated on **A** 12% SDS-polyacrylamide gels copolymerized with gelatin or **B** 10% SDS-polyacrylamide gels. In **A** the 30 kDa digestion bands in *lanes 1* and *2* were from *T. ocellata* and in *lanes 3* and *4* from *S. mansoni*. In **B** the gel was silver stained and showed a doublet of protein bands at 30 kDa for *T. ocellata* (*lane 1*) and *S. mansoni* (*lane 2*). Note that the digestion and silver-stained bands are stronger with *T. ocellata* than *S. mansoni*

EDTA, anti-pain and aprotinin (not illustrated). The K_i values for the four inhibitors were obtained from Dixon plots, which express K_m/V_{max} as a function of the inhibitor concentrations and where the K_i values are determined as the negative value of the intercept with the *x*-axis (Figs. 3A, B). K_i values for *T. ocellata* and *S. mansoni* are listed in Table 1.

The MW of serine protease activities was determined on gelatin-containing SDS-polyacrylamide gels and evidenced as digestion bands. As shown in Fig. 4A, the apparent MW was 28 kDa for both species. The intensity of the digestion band from *T. ocellata* was twice as high as that from *S. mansoni*. Silver staining of SDS-polyacrylamide gels without gelatin after electrophoresis of the CSs from both species showed a doublet band at 28 kDa (Fig. 4B). This doublet represents the CE in agreement with Darani et al. (1997), who showed that a rabbit antiserum against the CE recognized a doublet of antigens at 27–28 kDa in immunoblots.

This is the first biochemical description of serine protease activity in CSs from *T. ocellata*. The enzyme activity was inhibited by PMSF and aprotinin, which are selective inhibitors for serine proteases (Powers and Harper 1986; Brinkmann et al. 1997; Baciewicz 1998). Inhibition of the enzyme with anti-pain supports its trypsin-like activity (Nishikata et al. 1989; Kido et al. 1993). This is also the first comparison with the serine protease activity in CSs from *S. mansoni*. The MW of the enzyme from *T. ocellata*, the alkaline pH optimum, and the Ca^{2+} dependency all agree with the characteristics described by McKerrow et al. (1985) for the elastase of cercariae from *S. mansoni*. However, the specific enzyme activity in CSs from *T. ocellata* was always 2- to 3-fold higher than from *S. mansoni*. Similarly, CSs of *T. ocellata* gave stronger digestion bands on gelatin gels than CSs from *S. haematobium* and *S. japonicum* (Bahgat et al. 2001). We suggest that the proteinase characterized here from *T. ocellata* corresponds to enzymes, which were recently suspected, in analogy to human schistosomes, to facilitate the migration of bird schistosomes through the host's skin (Horak and Kolarova 2001).

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