Comparison of cysteine peptidase activities in *Trichobilharzia regenti* and *Schistosoma mansoni* cercariae

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SUMMARY

Cercariae of the bird schistosome *Trichobilharzia regenti* and of the human schistosome *Schistosoma mansoni* employ proteases to invade the skin of their definitive hosts. To investigate whether a similar proteolytic mechanism is used by both species, cercarial extracts of *T. regenti* and *S. mansoni* were biochemically characterized, with the primary focus on cysteine peptidases. A similar pattern of cysteine peptidase activities was detected by zymography of cercarial extracts and their chromatographic fractions from *T. regenti* and *S. mansoni*. The greatest peptidase activity was recorded in both species against the fluorogenic peptide substrate Z-Phe-Arg-AMC, commonly used to detect cathepsins B and L, and was markedly inhibited (>96%) by Z-Phe-Ala-CHN$_2$ at pH 4·5. Cysteine peptidases of 33 kDa and 33–34 kDa were identified in extracts of *T. regenti* and *S. mansoni* cercariae employing a biotinylated Clan CA cysteine peptidase-specific inhibitor (DCG-04). Finally, cercarial extracts from both *T. regenti* and *S. mansoni* were able to degrade native substrates present in skin (collagen II and IV, keratin) at physiological pH suggesting that cysteine peptidases are important in the pentration of host skin.

Key words: *Trichobilharzia*, *Schistosoma*, cercaria, cysteine peptidase, protease, penetration, DCG-04.

INTRODUCTION

Invasive larvae (cercariae) of schistosomatid trematodes infect their hosts by actively penetrating the skin. *Trichobilharzia regenti* is a bird (family Anatidae) schistosome with a unique route of migration; its larvae enter peripheral nerves and the spinal cord to reach the brain and ultimately the nasal cavity (Hrádková and Horák, 2002) evoking severe pathology (Horák *et al.* 1999; Kolářová *et al.* 2001). Although ducks are fully permissive to *T. regenti*, cercariae of this species are also able to invade mammals (including man), and can survive for a limited period of time causing severe dermatological and neurological pathologies related to host immune status (Kouřilová *et al.* 2004*a,b*; Horák and Kolářová, 2005). Repeated invasion can stimulate an allergic reaction in man, which is manifested as cercarial dermatitis (Horák and Kolářová, 2001; Horák *et al.* 2002). This disease is becoming an emerging public health problem in Europe (e.g. Bayssade-Dufour *et al.* 2002).

The mechanism of skin penetration is partially understood in some schistosome species (e.g.

Schistosoma mansoni and *S. japonicum*) and it is agreed that it involves the release of specific proteolytic enzymes (peptidases) (Dalton and Brindley, 1997; McKerrow and Salter, 2002; Whitfield *et al.* 2003; Ruppel *et al.* 2004; He *et al.* 2005). These peptidases are present in 2 groups of large penetration glands (post-acetabular and circumacetabular) filling almost two thirds of the cercarial body. After close attachment of the cercarial to host skin, the contents of these glands are released and the enzymes facilitate disruption of surface proteins and underlying tissues (for reviews see Horák *et al.* 2002; McKerrow *et al.* 2006).

Several cercarial serine peptidases have been described in *S. mansoni*, the best characterized of which is the 28 kDa or 30 kDa cercarial elastase (SmCE) (Landsperger *et al.* 1982; McKerrow *et al.* 1985; Marikovsky *et al.* 1988, 1990; Chavez-Olortegui *et al.* 1992; Salter *et al.* 2002). This chymotrypsin-like peptidase is localized in the circumacetabular glands and can cleave human skin elastin (McKerrow *et al.* 1985; Salter *et al.* 2000). However, the identity and function of cercarial cysteine peptidases is not well known but the presence of cathepsins L1 and B1 in *S. mansoni* has been reported (Dalton *et al.* 1996, 1997; Brady *et al.* 2000; Skelly and Shoemaker, 2001) these enzymes may be involved in the disruption of the outer keratinized layer of skin (Dalton *et al.* 1996, 1997). In bird schistosomes, 6 isoforms of cathepsin

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B1 (TrCB1) were reported from schistosomula of *T. regenti* (Dvořák *et al.* 2005). In this stage, these peptidases are localized in the gut and probably serve as digestive enzymes. Cathepsin B1 has also been recently identified in a cDNA library from sporocysts/cercariae of *T. regenti*, and its sequence is 100% identical to the schistosomular isoform TrCB1.1 (Dolečková *et al.* 2007).

In the present study, we show that the predominant peptidase activity in extracts of *T. regenti* cercariae is of the cysteine class, and has a similar substrate specificity, pH optimum and molecular size to a cysteine peptidase found in extracts of *S. mansoni* cercariae. The peptidases of both parasites are capable of degrading native keratin and collagens (types II, IV), and we show that they are cathepsin B-like enzymes.

MATERIALS AND METHODS

Parasites and reagents

Cercariae of *T. regenti* (laboratory strain) from intermediate host snails (*Radix peregra* s. lat.) were collected, washed twice in ice-cold 0·1 M phosphate-buffered saline (PBS) pH 7, and concentrated by centrifugation prior to storage at −80 °C. Live *S. mansoni* cercariae were obtained from 3 infected *Biomphalaria glabrata* snails donated by Dr Libuše Kolářová (Institute for Postgraduate Medical Education, Prague, CZ). Lyophilized *S. mansoni* cercariae were kindly provided by Professor Michael Doenhoff (School of Biological Sciences, University of Wales, UK). Eight-day-old schistosomula of *T. regenti* from duckling spinal cords were obtained as described by Dvořák *et al.* (2005). Extracts of cercariae (CE) and schistosomula (SE) were prepared by 2 cycles of sonication (7W, 30 s each, Vibracell-72405 100-W ultrasonicator, Bioblock Scientific, France) in 0·1 M PBS followed by centrifugation for 20 min at 16 000 *g* (4 °C). Protein concentration in supernatants was measured using the Bicinchoninic Acid Protein Assay (BCA-1, Sigma-Aldrich).

The DCG-04 probe for cysteine peptidase detection was a gift from Dr C. Caffrey and Dr D. Greenbaum, Sandler Center for Basic Research in Parasitic Diseases, UCSF, USA. All chemicals used were purchased from Sigma-Aldrich unless otherwise noted.

Fractionation of parasite extracts by chromatography

Anion-exchange chromatography was performed with the Bio-Logic system using a Mono Q column (Bio-Rad). Soluble *T. regenti* CE was first filtered using 0·22 μm Ultrafree-MC Sterile filtration device (Millipore) and then loaded (1·5 mg) onto a column equilibrated with 20 mM Bis-Tris-HCl buffer (Bis[2-hydroxyethyl]amino-tris[hydroxy-methyl]methane) pH 6·8. Elution was performed using a linear gradient of ionic strength (0–1 M NaCl in the same buffer) at the flow rate 1 ml/min. Fractions (300 μl) containing peptidase activity were further fractionated by gel permeation chromatography (GPC) using a Superdex 200 column (Amersham Pharmacia Biotech, USA).

T. regenti and *S. mansoni* CE (4 mg in 1 ml of 0·1 M PBS pH 7) were fractionated using a Superdex 75 column (Amersham Pharmacia Biotech, USA). Eluted proteins were collected in 80 fractions (300 μl/fraction) at a flow rate of 0·5 ml/min.

Fluorometric assays of enzyme activity

Peptidase activity was measured using fluorogenic (aminomethylcoumarin – AMC) peptide substrates (Bachem, Switzerland) each designed to assay specific peptidase activites: Z-Phe-Arg-AMC (FR; to assay cathepsins B and L), Z-Arg-Arg-AMC (RR; cathepsin B), Boc-Val-Leu-Lys-AMC (VLK; cathepsins B and L), Z-Arg-AMC (R; cathepsin H, aminopeptidase B, C), Z-Gly-Pro-Arg-AMC (GPR; trypsin-like), Z-Pro-Arg-AMC (PR; thrombin-like), Suc-Ala-Ala-Pro-Phe-AMC (AAPF; chymotrypsin-like), Boc-Leu-Gly-Arg-AMC (LGR; trypsin-like), Boc-Val-Pro-Arg-AMC (VPR; thrombin, trypsin) and Boc-Ala-Gly-Pro-Arg-AMC (AGPR; trypsin-like). Assays were carried out in 96-well black plates (Nunc, Denmark). Released free AMC was measured at excitation and emission wavelengths of 340–360 and 440–460 nm respectively in a Bio-Tek, Synergy HT fluorometer (Bio-Tek, USA) or Spex Fluoromax 3 (Jobin Yvon Horiba, France) continually for 120 min at 37 °C or RT. Activity against the fluorogenic substrates was screened in a broad pH range – in 0·1 M citrate-phosphate buffer pH 3–8 (for cysteine peptidase detection containing 5 mM DTT, or 5 mM L-cysteine) and in 0·1 M glycine buffer, pH 8·5–10·5. Monitoring of peptidase activity was started by addition of 200 μl/well of appropriate buffer with 50 μM peptide-AMC substrate to 2 μg of protein (CE), or 1 μg of protein (particular fraction).

The specificity of peptidase activity was investigated using a spectrum of differentiating inhibitors added to incubation buffers. Inhibitors of cysteine peptidases: 10 μM E-64 [N-trans-(epoxysuccinyl)-L-leucine 4-guanidinobutylamide], irreversible broad spectrum; 10 μM CA-074 [N-(L-3-*trans*-propylcarbamoyloxirane-2-carbonyl)-Ile-Pro-OH], irreversible selective inhibitor of cathepsin B (Towatari *et al.* 1991); 10 μM Z-Phe-Ala-CHN₂, irreversible inhibitor of cathepsins B and L (Dalton and Brindley, 1997); 10 μM calpain II inhibitor [Ac-Leu-Leu-Met-aldehyde], irreversible selective inhibitor of cathepsins L and B (Donkor, 2000).

Inhibitors of serine peptidases: 10 μM PMSF [phenylmethylsulfonyl fluoride], irreversible broad spectrum; elastatinal [N-(Na-Carbonyl-Cpd-Gln-Ala-al)-Leu], an irreversible specific inhibitor of neutrophil and pancreatic elastase but not other serine peptidases like trypsin or chymotrypsin (Bieth, 2004); 1·5 μM aprotinin from bovine lung, reversible broad spectrum.

Peptidase activity was also examined in the penetration gland secretory products (GSP) of live *T. regenti* and *S. mansoni* cercariae. Stimulation of penetration gland secretion was performed by praziquantel (Mikeš *et al.* 2005). Suspensions of live cercariae were placed onto microscope slides and incubated for 30 min with praziquantel (0·1 μg/ml in water; from 10 000 × stock solution in pure ethanol). Slides were then incubated with either 100 μM FR-AMC alone (30 min), or with 10 μM E-64 (15 min), followed by a mixture of 100 μM FR-AMC and 10 μM E-64 (30 min). The activity of released GSP was monitored by fluorescence microscopy (Olympus BX51).

Electrophoresis and zymography

The CE (5–20 μg of protein) and chromatographic fractions of *T. regenti* and *S. mansoni* were separated by SDS-PAGE (MiniProtean-3 apparatus, Bio-Rad) in 10% and 12% gels, or in 4–20% gradient gels. Peptidase activities were assayed by zymography in gels co-polymerized with 0·1% gelatin. Samples were mixed with a non-reducing sample buffer, or reducing buffer (10 mM DTT), and allowed to stand at room temperature for 10 min prior to loading. Following electrophoresis, zymographic gels were washed 2 × 10 min in either 0·1 M citrate-phosphate buffer, pH 3·5 to 8, or glycine buffer pH 9 and 10 (both with/without 10 μM E-64, or 1·5 μM aprotinin) containing 2·5% Triton X-100 and then 1 × 10 min in an appropriate Triton-free buffer. Overnight incubation was carried out in the same buffers (in the case of citrate-phosphate buffer, 10 mM L-cysteine was added). All gels were stained with Coomassie Brilliant Blue R-250, or using a Silver Stain Kit (Bio-Rad).

Hydrolysis of macromolecular substrates

Individual fractions (0·75 μg of protein in 5 μl) were mixed with collagen type II from bovine nasal septum, type IV from human placenta, or keratin from human epidermis (all 10 μg in 10 μl of 0·1 M PBS pH 7). Collagenase from *Clostridium histolyticum* (0·5 μg in 5 μl) was used as a positive control. The mixtures were incubated for 6 h at 37 °C and then separated by SDS-PAGE in 12% polyacrylamide gels for detection of digestion products.

Ligand blotting with DCG-04

DCG-04 is a biotinylated analogue of the irreversible Clan CA cysteine peptidase inhibitor E-64 which covalently binds to the active site of cysteine peptidases (Greenbaum *et al.* 2000). *T. regenti* and *S. mansoni* CE, *T. regenti* fraction 7′ that has the highest cysteine peptidase activity, and *T. regenti* SE (2 μg of total protein each) were incubated for 1 h with 5 μM DCG-04 in 0·1 mM citrate-phosphate buffer, pH 6·0, containing 5 mM DTT. Controls were pre-incubated with 100 μM cysteine peptidase inhibitors E-64, or CA-074. After SDS-PAGE, proteins were transblotted onto nitrocellulose membrane (1 h, 1·5 mA/cm²) and blocked for 1 h in 5% non-fat milk (Bio-Rad Blotting Grade Blocker) in 20 mM Tris-buffered 0·15 M saline, pH 7·8 (TBS; Tris[hydroxymethyl]aminomethane) containing 0·05% Tween-20 (TBS-T). The membranes were washed 3 × 5 min in TBS-T and then incubated with streptavidin-HRP (2 μg/ml in 1% non-fat milk in TBS-T) for 30 min and washed again 3 × 5 min in TBS-T (Mikeš and Man, 2003). The membrane was developed using the Opti-4CN™ Substrate Kit (Bio-Rad).

RESULTS

Fluorometric enzyme assays with T. regenti and S. mansoni cercarial extracts, their fractions and gland secretion products

Soluble CE were screened for peptidase activities, and particularly for the presence of cysteine peptidases (e.g. cathepsins B and L). The optima for both *T. regenti* and *S. mansoni* cysteine peptidase activities in the presence of FR substrate were between pH 4·5 and 5 (Fig. 1A). The level of activity was 3·4 times lower for *T. regenti* compared to *S. mansoni*. Peptidolytic activities of CEs with other substrates were minor at this pH (Fig. 2; pH 4·5).

Trypsin-like serine peptidase activity was also noted in *S. mansoni* CE (rank order GPR > VPR > LGR > AGPR) (Fig. 2; pH 10), but only negligible activity was detected for *T. regenti* CE at the strongly alkaline values of the pH optimum 10–10·5 (Fig. 2; pH 10 and Fig. 1B). Chymotrypsin-like activity was demonstrated by slight cleavage of AAPF substrate in *S. mansoni* CE only (Fig. 2; pH 10). In both species, cysteine peptidase activities predominated over serine peptidase activities.

Both *T. regenti* and *S. mansoni* CE were fractionated by GPC on the Superdex 70 column into 80 fractions, yielding 0·15–0·19 mg/ml protein per 300 μl fraction (Fig. 3). The position of *T. regenti* fractions with the greatest cysteine peptidase activities (Ct1, 2, 3; peak of activity in fraction Ct2; Fig. 3A) corresponded to the positions of active fractions of *S. mansoni* CE elution profiles (Cs1, 2, 3; peak of activity in fraction Cs2; Fig. 3B). The fractions
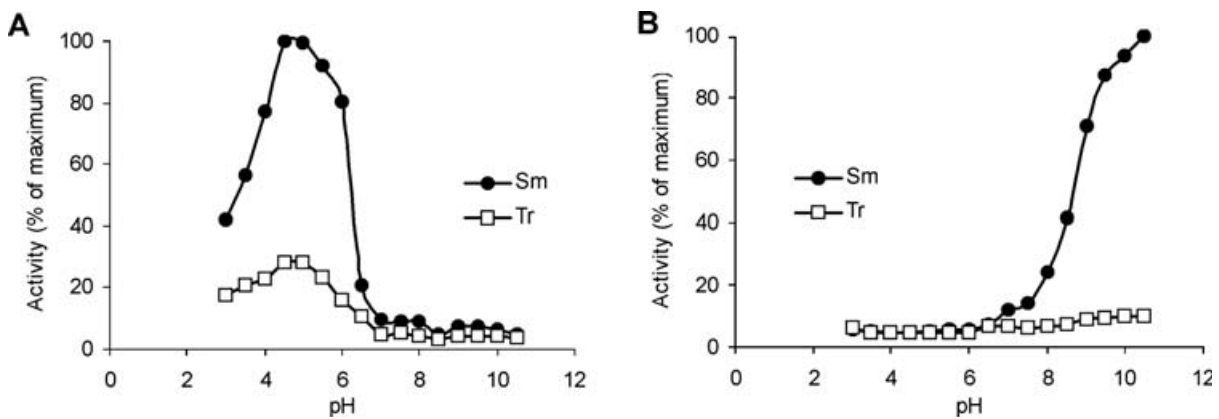
Fig. 1. The pH profile of cysteine peptidase-like and serine peptidase-like activities in CE of *Trichobilharzia regenti* and *Schistosoma mansoni*. Cysteine peptidase-like activity was assayed using the fluorogenic peptide substrate FR (A). Serine peptidase-like activity was assayed using GPR substrate (B). Activity was measured in 0·1 M citrate-phosphate buffer, pH 3–8, and in 0·1 M glycine buffer, pH 8·5–10·5, for 120 min at 37 °C.
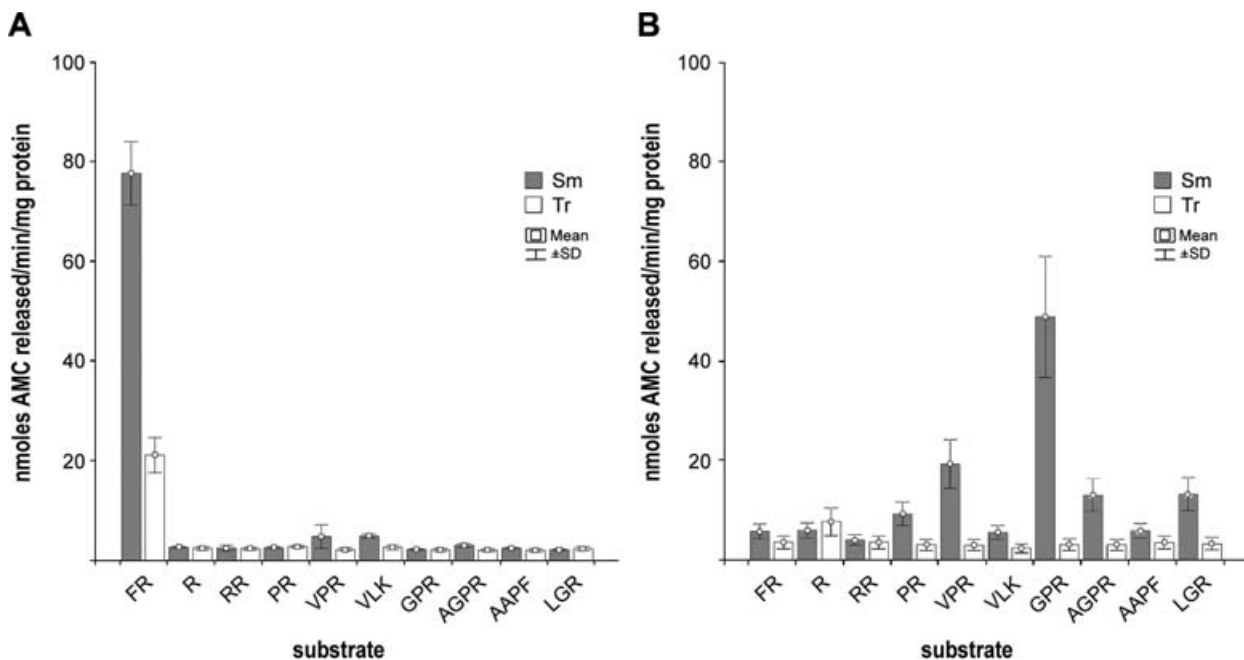

Fig. 2. Peptidase activity of CE from *Trichobilharzia regenti* (Tr) and *Schistosoma mansoni* (Sm). Assays performed using fluorogenic substrates in 0·1 M citrate-phosphate, buffer pH 4·5, in the presence of 5 mM L-cysteine (A), or 0·1 M glycine buffer, pH 10 (B). The activity on each substrate is expressed as nmoles AMC released/min/mg protein. Activity was measured for 120 min at 37 °C.: FR, Z-Phe-Arg-AMC; R, Z-Arg-AMC; RR, Z-Arg-Arg-AMC; PR, Z-Pro-Arg-AMC; VPR, Boc-Val-Pro-Arg-AMC; VLK, Boc-Val-Leu-Lys-AMC; GPR, Z-Gly-Pro-Arg-AMC; AGPR, Boc-Ala-Gly-Pro-Arg-AMC; AAPF, Suc-Ala-Ala-Pro-Phe-AMC; LGR, Boc-Leu-Gly-Arg-AMC.

Ct2 and Cs2 were able to cleave 4 fluorogenic peptide substrates (rank order FR>>VLK>RR>R) at pH 4·5. The activity against FR substrate in *T. regenti* Ct2 fraction increased 2·5 times compared to CE. This implies a successful partial purification of a cysteine peptidase.

Fractions of *T. regenti* CE obtained after anion-exchange FPLC were also screened for cysteine peptidase activities. Fraction 18 showed the highest preference for the cathepsin B and L substrate FR (Fig. 4A). This fraction was further fractionated

by GPC (Superdex 200 column). Fractions 6′ and 7′ from GPC expressed activity with the FR substrate (Fig. 4B). In later experiments only fraction 7′ was used because of its much higher peptidase activity.

Using FR substrate, peptidase activity was also detected in *T. regenti* and *S. mansoni* praziquantel-stimulated GSP (Fig. 5B). This was visible as a fluorescent cloud adjacent to penetration gland openings in front of the head organ. The activity was inhibited by 10 μM E-64 (Fig. 5D).
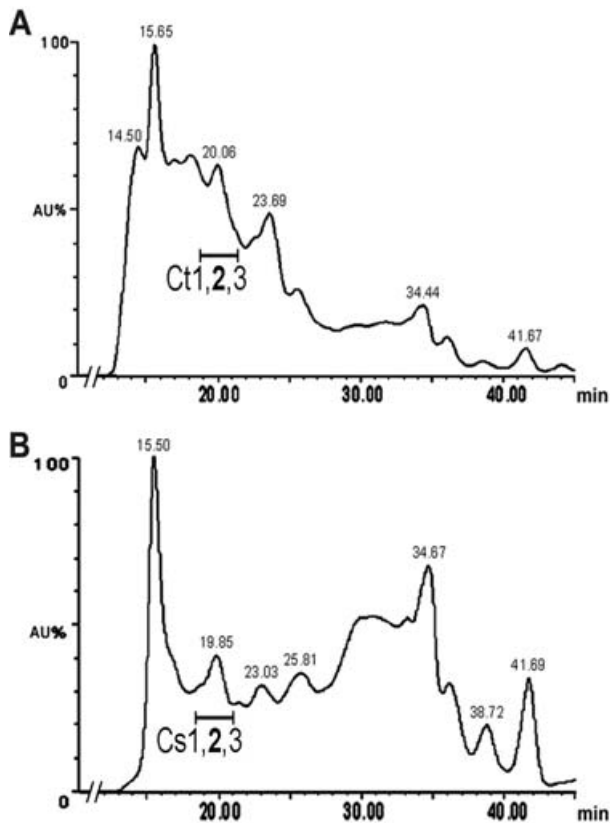
Fig. 3. Elution profile from gel permeation chromatography (Superdex 75 column) of *Trichobilharzia regenti* (A) and *Schistosoma mansoni* (B) CE. Numbers above the peaks mark the elution time. The bars under the peaks indicate the greatest cysteine peptidolytic activity of fractions against FR (*T. regenti* Ct1, 2, 3 and *S. mansoni* Cs1, 2, 3). The most active fractions Ct2 and Cs2 are in bold.

Inhibition assays

The peptidase activity of the chromatographic fractions and whole soluble CE was screened using a panel of differentiating peptidase inhibitors (Table 1). Z-Phe-Ala-CHN$_2$ was the most potent inhibitor ($>96\%$ inhibition) for all samples (Ct2, Cs2, TrCE, and SmCE) as tested by the FR substrate at pH 4·5. Other cysteine peptidase-specific inhibitors were also highly effective and caused inhibition of at least 85%. On the other hand, the serine peptidase-specific inhibitors aprotinin and PMSF only inhibited peptidase activity by 20%, although elastatinal caused 70% inhibition.

Gelatinolytic activity of T. regenti
and S. mansoni *samples*

Gelatin gels of *T. regenti* and *S. mansoni* CE and fractions (Ct1, 2, 3; Cs1, 2, 3) at pH 4·5 showed lysis in the regions 25–37 kDa (Fig. 6). This agrees with the result showing that fractions 18 and 7′ contained molecules with peptidase activity around 30 kDa (see Fig. 4). Cysteine peptidase activities of the different parasite preparations were significantly inhibited by incubating the gelatin gels overnight with 10 μM E-64 at pH 4·5 (Fig. 4 and Fig. 6).

Conversely at pH 10, gelatinolytic activity of CE from both schistosome species was localized in the area $\geqslant 45$ kDa (Fig. 6). Aprotinin significantly inhibited the serine peptidase activity of CE in this area (Fig. 6).

Degradation of keratin and collagen substrates

The native substrates, keratin and collagen (types II, IV), were degraded by particular fractions Ct2, Cs2 of *T. regenti* and *S. mansoni*, after overnight incubation at neutral pH (Fig. 7). The pattern of hydrolysis products differed between the two species.

Cysteine peptidase active site-labelling by DCG-04

For *T. regenti*, incubation with the DCG-04 probe led to the detection of a prominent 33 kDa band in CE, as well as in chromatographic fraction 7′ (with the highest cysteine peptidase activity) (Fig. 8). A similar 33/34 kDa band doublet was recorded in *T. regenti* SE. In *S. mansoni* CE, the band migrated at aproximately 33–34 kDa. Controls without DCG-04 and with E-64 or CA-074 pre-incubated sample showed no reaction in these regions.

DISCUSSION

The study described here constitutes a comparative analysis of the peptidases released by *T. regenti* and *S. mansoni* focussing upon cercarial cysteine peptidases. *S. mansoni* was used in order to evaluate the level of activity in *T. regenti*. Although we would be able to collect appropriate amounts of penetration gland products of *T. regenti* cercariae (Mikeš *et al.* 2005), we did not dispose of enough live *S. mansoni* cercariae. Therefore, cercarial protein extracts of both parasites were employed in this study. We were aware of the possible appearance of non-gland peptidases in the samples, however, this cannot be excluded even when working with cercarial penetration gland secretions (Knudsen *et al.* 2005; Mikeš *et al.* 2005).

General screening for peptidase activity initially compared selected fluorogenic substrates known to reveal the presence of various trematode peptidases, especially cathepsins B and L and *S. mansoni* elastase. Whole cercarial extracts (CE) were tested for their activity against specific substrates and at different pH optima. The preference for cysteine peptidase substrate FR was similar in CE of both *T. regenti* and *S. mansoni*, although the level of peptidase activity at the optimum of pH 4·5 was much greater for the latter species. It is not clear whether this was due to subtle differences in affinity
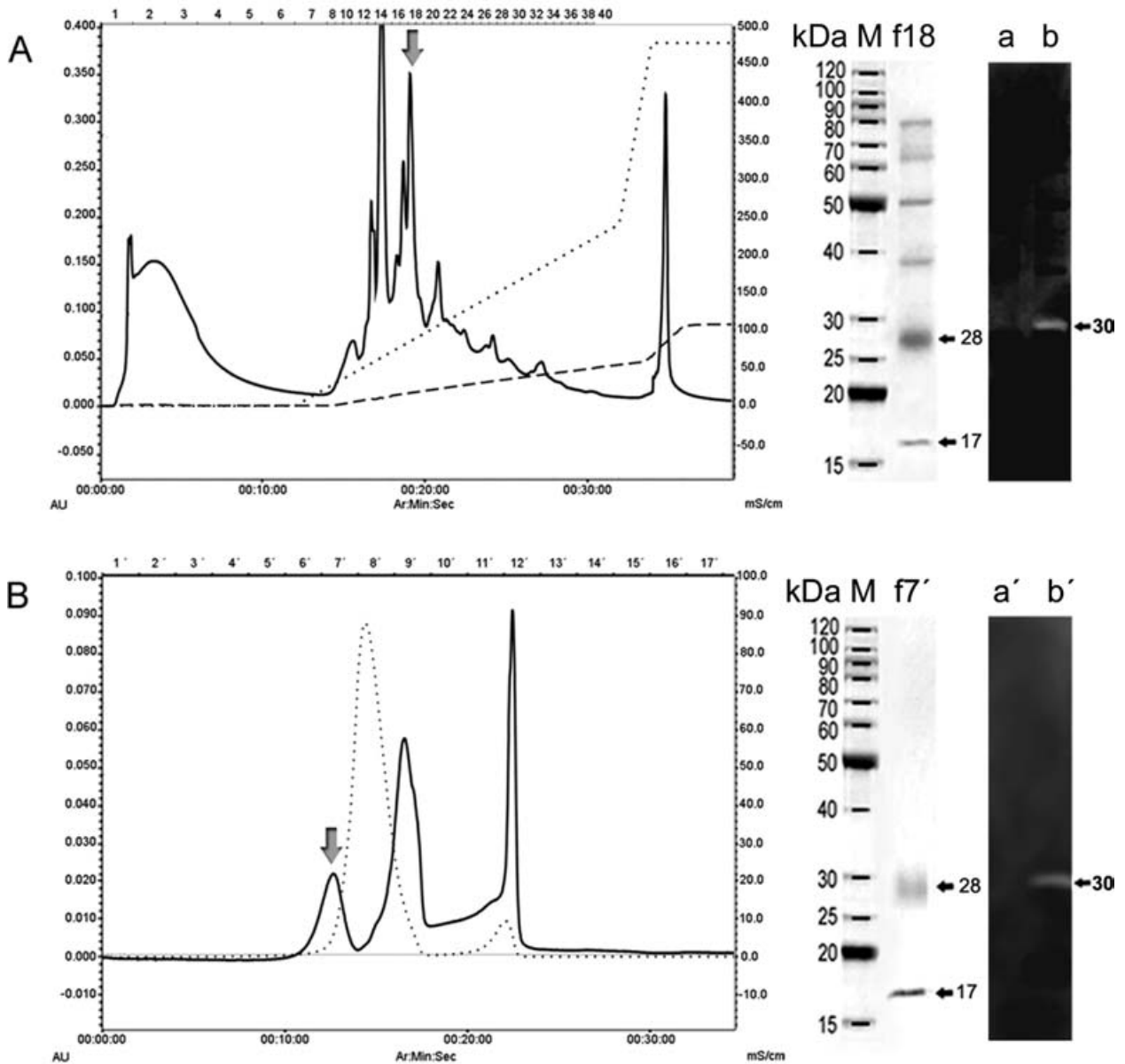
Fig. 4. Elution profile from ion-exchange FPLC (A) and subsequent gel permeation chromatography (B) of
Trichobilharzia regenti CE. SDS-PAGE (12% gel) of fractions with the highest cysteine peptidase activity (f18, f7′).
Shaded arrows show the fractions 18 (A) and 7′ (B). Dashed line, panel (A), conductivity. Dotted line, panel (A),
theoretical gradient of salt. Dotted line, panel (B), conductivity. Lanes M, molecular weight marker. Lane f18, fraction
18 eluted in anion-exchange FPLC. Lane f7′, fraction 7′ eluted in gel permeation chromatography. Lanes a, b and a′, b′,
zymographic analysis of fractions 18 and f7′, respectively. Lanes b, b′, overnight incubation of the gelatin gels in 0·1 M
citrate-phosphate buffer pH 4·5 without inhibitor. Lanes a, a′, overnight incubation in 0·1 M citrate-phosphate buffer,
pH 4·5, with 10 μM E-64. Both gels were stained with Coomassie Brilliant Blue R.

for FR substrate or because of lower cysteine pepti-
dase content in the case of *T. regenti* CE.

Trematode cysteine peptidases generally exhibit
their activity between pH 4 and 10 (Dalton and
Brindley, 1997; Caffrey *et al.* 2002; Sajid and
McKerrow, 2002) and it is known that *in vitro* the
pH optima of *S. mansoni* cysteine peptidases are
shifted to acid pH (e.g. SmCB1 – pH 6·0;
SmCB2 – pH 5·0–5·5; SmCL1 – pH 6·5; SmCL2 –
pH 5·35; for reviews see Caffrey and McKerrow

(2004) and Dalton *et al.* (2004)). Similarly, schisto-
somular cathepsin B1 of *T. regenti* expressed optimal
activity against FR substrate at pH 4·5–5·5 (Dvořák
et al. 2005). Serine peptidase optima, on the
other hand, generally occur at basic pH values (e.g.
S. mansoni cercarial elastinolytic protease – pH 8–10;
McKerrow *et al.* (1985); *S. mansoni* cercarial
elastase – pH > 9; Salter *et al.* (2000); for review see
Dalton and Brindley (1997)). Our findings fully
correspond with these results, although it should be

Table 1. *The effect of inhibitors on peptidolytic activity of* Trichobilharzia regenti *and* Schistosoma mansoni *cercarial extracts and fractions using Z-Phe-Arg-AMC substrate*

(Activity was measured in 0·1 M citrate-phosphate buffer pH 4·5, in the presence of 5 mM L-cysteine. Values are means of 3 independent triplicate assays with standard deviations ($\pm$ SD).)

	Inhibition (%)			
Inhibitor	TrCEs[a]	Ct2[b]	SmCEs[c]	Cs2[d]
E-64 (10 μM)	97·2 ($\pm$0·6)	91·4 ($\pm$2·1)	95·6 ($\pm$1·6)	96·1 ($\pm$1·1)
CA-074 (10 μM)	95·1 ($\pm$0·4)	88·3 ($\pm$4·2)	85·7 ($\pm$5·8)	89·8 ($\pm$1·2)
Z-Phe-Ala-CHN$_2$ (10 μM)	99·3 ($\pm$0·2)	96·5 ($\pm$1·0)	97·6 ($\pm$0·9)	99·2 ($\pm$0)
Calpain II (10 μM)	96·4 ($\pm$1·7)	98·5 ($\pm$0·6)	94·3 ($\pm$1·3)	91·5 ($\pm$3·6)
Aprotinin (1·5 μM)	21·6 ($\pm$5·2)	14·9 ($\pm$3·6)	0·7 ($\pm$0·1)	2·5 ($\pm$0·2)
PMSF (10 μM)	23·8 ($\pm$1·3)	11·3 ($\pm$0·8)	0·90 ($\pm$0·1)	4·1 ($\pm$0·9)
Elastatinal	89·9 ($\pm$5·1)	71·4 ($\pm$4·4)	90·1 ($\pm$2·8)	78·9 ($\pm$3·5)

[a, c] Cercarial protein extracts of *T. regenti* and *S. mansoni*.
[b, d] *T. regenti* and *S. mansoni* chromatographic fractions with the highest activity against Z-Phe-Arg-AMC substrate.
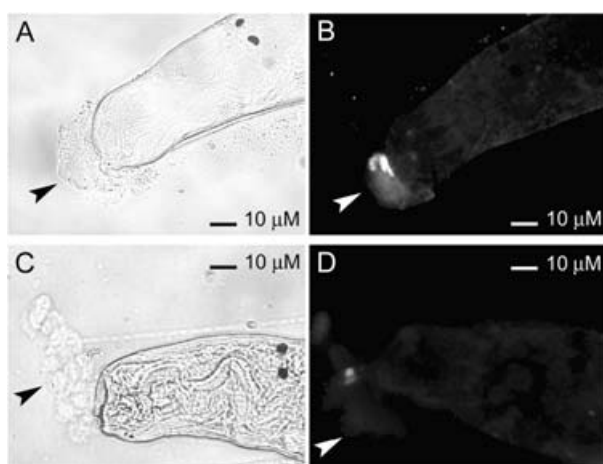

Fig. 5. Cysteine peptidase activity in the penetration gland secretions of *Trichobilharzia regenti* cercariae. Penetration gland secretion was induced by adding praziquantel to a suspension of cercariae. Released excretory/secretory products were incubated with FR substrate (B) or with E-64 inhibitor followed by FR substrate (D). Released products in front of cercarial head organ, observed under bright field conditions (A, C black arrows). Degradation of peptidyl substrate by gland peptidases revealed by fluorescence microscopy (B, white arrow). Inhibition of this activity was recorded in control containing E-64 inhibitor (D, white arrow). Analogous results were obtained with *S. mansoni* cercariae under the same experimental conditions (not shown).

noted that we were unable to detect significant quantities of serine peptidases in CE of *T. regenti*.

Inhibition studies showed that the general cysteine peptidase inhibitor E-64 and the cathepsin B and L inhibitor Z-Phe-Ala-CHN$_2$ had significant effects on peptidase activity against the FR substrate at pH 4·5. The cathepsin B-selective inhibitor CA-074 also resulted in a comparable level of inhibition. These

results imply that the major peptidase activities in *T. regenti* CE are of cysteine peptidase origin – most likely cathepsin B and, to a certain degree, cathepsin L. The relatively high inhibitory effect of elastatinal on cysteine peptidase activity with FR substrate was unexpected and its action is questionable. Elastatinal is usually regarded as a specific inhibitor of pancreatic and neutrophil elastases (serine peptidases). Considering its structure [N-(Na-Carbonyl-Cpd-Gln-Ala-al)-Leu] and the fact that Z-Phe-Ala-diazomethylketone was the best inhibitor, it is likely that the aldehyde on Ala$_2$ of elastatinal inhibits the cysteine peptidase activity when situated in P1 position. The data presented here with FR substrate and the inhibitors corroborate previously reported results on cathepsins B or L. These enzymes are ubiquitous in somatic extracts or excretory/secretory products of trematodes including *S. mansoni* larvae and adults (Dalton *et al.* 1996; Caffrey *et al.* 1997; Dalton *et al.* 1997; Brady *et al.* 2000; Sajid *et al.* 2003) or *T. regenti* schistosomula (Dvořák *et al.* 2005).

Screening for cysteine peptidase activity in fractionated CE (fractions Ct2, Cs2 and 7′) of *T. regenti* revealed results consistent with those obtained with *S. mansoni*. The 2 protein bands (17 and 28 kDa) detected in *T. regenti* cysteine peptidase active fraction 7′ were characterized by mass spectrometry methods (MALDI-TOF MS). Their tryptic peptides were *de novo* sequenced (LC MS/MS, ion trap). However, none of the obtained peptide sequences (from the 17 and 28 kDa bands) aligned with known peptidase sequences in databases (not shown). This implies that the cysteine peptidase activity was produced by a minute amount of a highly active enzyme (not detectable in the polyacrylamide gel). This is consistent with the proteomic surveys of *S. mansoni* cercariae performed by Knudsen *et al.* (2005) and Curwen *et al.* (2006), who did not find cysteine
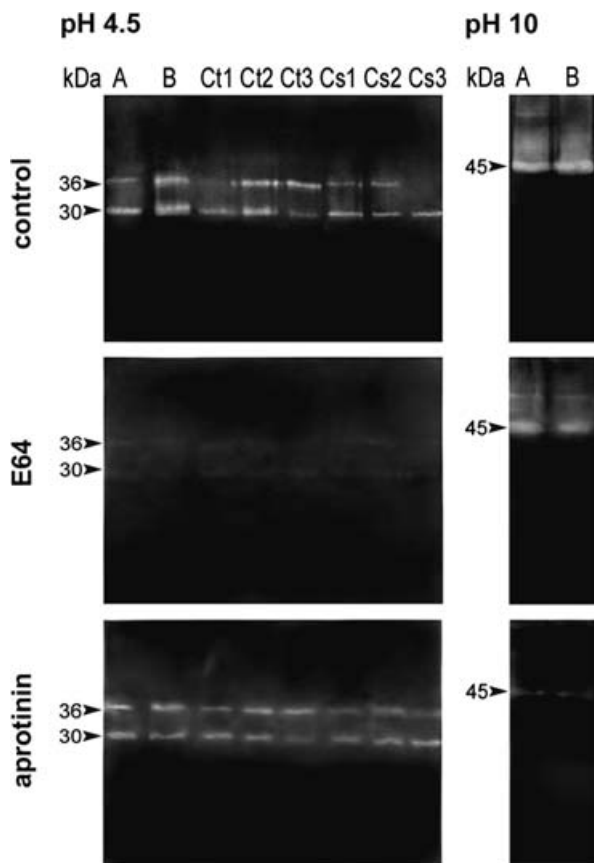
Fig. 6. Analysis of proteolytic activity in cercarial protein extracts and fractions of *Trichobilharzia regenti* and *Schistosoma mansoni* by zymography (12% polyacrylamide gel co-polymerized with 0·1% gelatin). Lanes A, CEs of *T. regenti* (10 μg of protein per lane). Lanes B, CEs of *Schistosoma mansoni* (10 μg of protein per lane). Ct1, 2, 3 and Cs1, 2, 3, fractions of *T. regenti* and *S. mansoni* with the highest cysteine-like peptidase activity (1 μg of protein per lane; see Fig. 3). Control, the gels after SDS-PAGE incubated overnight in 0·1 citrate-phosphate buffer (pH 4·5) and 0·1 M glycine buffer (pH 10; 37 °C). Aprotinin and E64, gels incubated overnight in 0·1 M citrate-phosphate buffer (pH 4·5) and 0·1 M glycine buffer (pH 10; 37 °C) with the respective inhibitor.
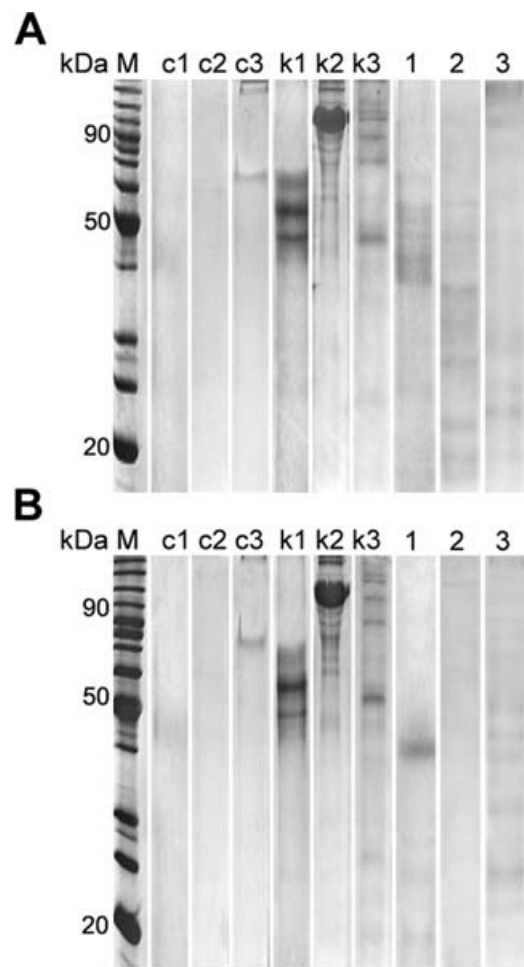

Fig. 7. Degradation of keratin, collagen II and collagen IV by *Trichobilharzia regenti* Ct2 fraction (A) and *Schistosoma mansoni* Cs2 fraction (B). Samples were separated by SDS-PAGE in 12% polyacrylamide gel for detection of digestion products. Lane M, molecular weight marker. Lanes c1, c2, c3, control collagenase (0·5 μg in 5 μl) with keratin, collagen II, collagen IV. Lanes k1, k2, k3; keratin, collagen II, collagen IV alone (10 μg in 10 μl). Lanes 1, 2, 3; fractions Ct2 or Cs2 (0·5 μg of protein in 5 μl) incubated with keratin, collagen II, collagen IV. Collagenase and fractions were incubated with keratin, collagen II, collagen IV (10 μg in 10 μl) in 0·1 M PBS, pH 7, for 6 h at 37 °C, prior to electrophoresis.

peptidases among penetration gland-secreted proteins. Nevertheless, cysteine peptidase activity was clearly demonstrated in cercarial GSP of both species in our experiments.

The cleavage of the GPR substrate was negligible in the case of *T. regenti* compared to *S. mansoni*. Hydrolysis of this substrate is usually related to serine peptidase activity (Zimmerman *et al*. 1977; Dalton *et al*. 1997; Bahgat and Ruppel, 2002) specifically at alkaline pH values 8–10 (Dalton and Brindley, 1997). However, it is known that trypsin-like peptidases preferentially cleave this substrate compared to chymotrypsin-like peptidases including cercarial elastase from *S. mansoni* (Salter *et al*. 2000, 2002). Therefore, our measurements may result from contamination of *S. mansoni* CE by trypsin-like

peptidases of snail origin (Salter *et al*. 2000). Nevertheless, the presence of peptidases active against the AAPF substrate confirmed the presence of *S. mansoni* cercarial elastase in CE. The virtual absence of a serine peptidase activity in *T. regenti* CE indicates that an elastase orthologue is not present in *T. regenti*. This reinforces the failure to reveal an elastase-like enzyme in this species using anti-*S. mansoni* elastase antibodies (Mikeš *et al*. 2005) or molecular techniques (Dolečková *et al*. 2007). At this point, the *T. regenti* penetration mechanism is reminiscent of that in the human schistosome *S. japonicum*, where no cercarial elastase was reliably
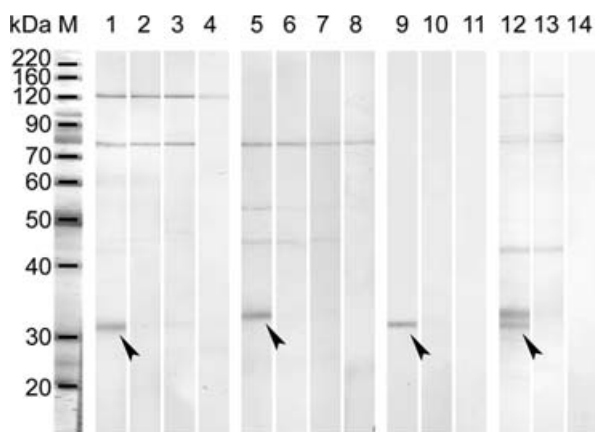
Fig. 8. Binding of DCG-04, a cysteine peptidase-specific probe, to the peptidases in CE and SE extracts of *Trichobilharzia regenti*, chromatographic fraction 7′ of *T. regenti* and CE of *Schistosoma mansoni*. Lane M, molecular weight marker. Lanes 1–4, *T. regenti* CE, lanes 5–8, *S. mansoni* CE, lanes 9–11, *T. regenti* chromatographic fraction 7′, lanes 12–14, *T. regenti* SE. Lanes 1, 5, 9 and 12, positive reaction with DCG-04. Lanes 2, 6, 10 and 13, no binding of DCG-04, reaction blocked by cysteine peptidase inhibitor E-64. Lanes 3 and 7, no binding of DCG-04, reaction blocked by selective cathepsin B inhibitor CA-074. Lanes 4, 8, 11 and 14, controls of non-specific avidin-Px binding without DCG-04; 2 μg of protein per lane, 5 μM DCG-04. Arrows show the detected cathepsin B in *T. regenti* CE and fraction 7′ (33 kDa, lanes 1 and 9), in *T. regenti* SE (33/34 kDa, lane 12) and in *S. mansoni* CE (33–34 kDa, lane 5).

identified yet (e.g. Fan *et al.* 1998; Chlichlia *et al.* 2005).

In zymographs of fraction 7′, the ~ 30 kDa lytic band is possibly the same as that detected at pH 4·5 in *T. regenti* CEs and in the Ct2 fraction. This lytic band apparently did not match the sequenced 28 kDa band in fractions 7′ and 18. The ~ 30 kDa band within fraction 7′ most likely corresponds to the ~ 33 kDa band detected after incubation of *T. regenti* CE, fraction 7′ and schistosomular extract with DCG-04. The difference of ~ 3 kDa in size could be caused by changed mobility of the protein caused either by the covalently bound probe in case of ligand blotting, or by the gelatin content in the case of zymographic gels.

Although many other researchers have reported analogous gelatinolytic patterns caused by peptidase activities in *S. mansoni* CE (McKerrow *et al.* 1985; Marikovsky *et al.* 1988; Chavez-Olortegui *et al.* 1992; Dalton *et al.* 1997; Bahgat *et al.* 2002), only one group has analysed the proteolytic activity of *T. regenti* enzymes (Dvořák *et al.* 2005; Mikeš *et al.* 2005). There is also limited information on the role of cysteine peptidases in cercarial penetration, although it is speculated that they may aid the entry of schistosome cercariae through the outer keratinized

layer of the skin (Dalton *et al.* 1997). Our results illustrating degradation of keratin and collagen (type II and IV) by cercarial cysteine peptidases support this theory. Indeed, the positive reaction of DCG-04 with *T. regenti* and *S. mansoni* proteins at ~ 33 kDa and 33–34 kDa, respectively, proved the presence of cysteine peptidases. The inhibition of these reactions by CA-074 confirmed that they are cathepsins B. In schistosomula of *T. regenti*, the 33/34 band doublet corresponding to the ~ 33 kDa band of cercariae identified by DCG-04, has previously been determined as cathepsin B1 (TrCB1 – Dvořák *et al.* 2005). The occurence of TrCB1 in cercariae of this species is also supported by the identification of its sequence in the cDNA library of cercarial germ balls (Dolečková *et al.* 2007). Thus, we have demonstrated peptidases of cathepsin B-type in the penetration glands of cercariae of 2 schistosome species. The ability of these cysteine peptidases to hydrolyse skin proteins supports their role in host invasion.

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