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Setaria cervi: *in vitro* released collagenases and their inhibition by *Wuchereria bancrofti* infected sera

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Abstract

In vitro released products of adult *Setaria cervi* females, microfilariae and extracts showed considerable amounts of collagenase activity. On the basis of per mg protein released *in vitro*, the products of both microfilariae and adult females exhibited comparable activity but this was much higher than that of extract of microfilariae and adult females. Two collagenase enzymes with molecular masses of 50 kDa and 70 kDa were separated using DEAE-sepharose CL6B and Sephadex G-100 column chromatography. The 50 kDa and 70 kDa collagenase exhibited pH optima of 5.2 and 7.0, respectivly. Considering specific activity, the 50 kDa enzyme was found to contribute about ten times more collagenase activity as compared to the 70 kDa enzyme. An inhibition study revealed obvious differences between them. Thiol group inhibitors such as *N*-ethylmaleimide and leupeptin inhibited the 50 kDa enzyme but this was strongly activated by dithiothreitol, a thiol group stabilizer. Alternatively, the 70 kDa enzyme showed a sensitivity to a metal chelator and a serine group inhibitor indicating its metalloserine protease nature. The antifilarial drug diethylcarbamazine did not demonstrate any inhibition under *in vitro* conditions. Both enzymes were significantly inhibited by antibody IgG separated from *Wuchereria bancrofti* infected human sera, showing a possible immunoprotective role.

Introduction

The mechanism by which filarial worms penetrate and migrate within host tissues is unclear. Chandler (1932) suggested that these parasites might secrete proteases for this purpose and consequently proteases have been extensively explored in many helminths and protozoans but how the non-specific proteases interact is not clearly understood (Lewart & Lee, 1954; Munoz *et al.*, 1982; Dresden *et al.*, 1985; Pino-Heiss *et al.*, 1985; Thomas *et al.*, 1994). The presence of chondromucoprotein and elastin digesting enzymes in extracts of *Schistosoma mansoni* cercariae suggests that these enzymes are used to penetrate the tissues of susceptible hosts (Dresden & Asch, 1972; Mckerrow *et al.*, 1985). Enzymes with a similar role have been detected in extracts of *Strongyloides ransomi* larvae (Dredsen *et al.*, 1985). Petralanda *et al.* (1986) working on onchocerciasis and brugian filariasis have also identified enzymes in adult worm extracts and in live microfilariae (mf) of *Onchocerca volvulus* and *Brugia malayi* with collagenase activity. As collagen and elastin are major constituents of the extracellular matrix, the presence of collagenase in the filarial worms strongly suggests its involvement in penetration of host tissues by these parasites. Perhaps the most intriguing role proposed for proteases is that of evading the host immune response. Proteases of *Schistosoma mansoni* schistosomula and *Dirofilaria immitis* microfilariae have been shown to cleave IgG (Auriault *et al.*, 1981; Tamashiro *et al.*, 1987). But the host immune system responds protectively to an enzyme present in *Babesia bovis* infected erythrocytes and proteinases released by parasitic larval stages of *Ascaris suum* (Commins *et al.*, 1985; Knox & Kennedy, 1988).

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As the filarial parasite *Setaria cervi* inhabits the visceral cavity of water buffaloes, the parasite must pass through the tissues (which largely possess collagen and elastin in extracellular matrix) lining the cavity. The aims of the present study are therefore to determine whether or not collagenases are present in adult *S. cervi*, the microfilarae and also the excretory products of both adult female and microfilariae and to investigate whether secreted enzymes are inhibited by IgG separated from *Wuchereria bancrofti* infected human sera.

Materials and methods

Chemicals, human haemoglobin, azocasein, collagen, elastin-orcein, ethylene diaminetetra-acetic acid (EDTA), N-ethylmaleimide (NEM), phenylmethylsulphonyl fluoride (PMSF), and leupeptin were purchased from the Sigma Chemical Co., St Louis, Missouri, USA. Sephadex G-200, diethyl-amino-ethyl-sepharose and dithiothreitol (DTT) were products of Pharmacia, Uppsala, Sweden. All other chemicals used were of an analytical grade purity.

Collection of parasites and excretory–secretory products

Adult *Setaria cervi* worms, from freshly slaughtered water buffaloes, were collected in Kreb's Ringer bicarbonate buffer from the local abattoir. Microfilariae were obtained by dissecting gravid females, and uteri containing mf were kept in Ringer's solution at 37°C for 1 h. The mf released in the medium were collected by mild centrifugation. For collection of excretory–secretory products, mf were maintained in Kreb's Ringer solution with 1% glucose for 2 h at 37°C under sterilized conditions. After 2 h, mf were removed from the culture medium by gentle centrifugation. Pooled culture media were concentrated and dialysed. Excretory–secretory products of adult *S. cervi* females were obtained by incubating ten worms in 10 ml Ringer's solution. After 2 h incubation worms were removed and the Ringer's solution containing excretory–secretory products was centrifuged and the supernatant used for the collagenase assay.

Preparation of adult S. cervi *and microfilarial extracts*

Ten percent adult *S. cervi* extracts were prepared by homogenizing females in phosphate buffered saline at 4°C and centrifuging at 10,000 rpm for 30 min. The supernatant was used for further studies.

Separation of IgG from human sera

Night thick blood smears from people living at Chiraigaon, a rural area near Varanasi, Uttar Pradesh, India, were prepared and examined for mf. The blood of mf-positive persons was taken with their consent and the serum separated. Immunoglobulins were precipitated from normal and *W. bancrofti* infected human sera using cold saturated ammonium sulphate solution (Heide & Schwick, 1978). IgG was separated by DEAE-cellulose column chromatography after Fahey & Terry (1978).

Assay of protease activity

Reaction mixtures comprised 5 mg solid collagen type V from bovine Achilles tendon, 20 μl (2.27 μg) enzyme from different stages of *S. cervi* and *in vitro* released products of adult female or mf of *S. cervi* in 980 μl buffer. After 20 min of incubation at 37°C, 1.0 ml 10% trichloroacetic acid was added to stop the reaction, left for 1 h at room temperature and then centrifuged. The supernatant was taken for estimating digested protein using the method of Lowry *et al.* (1951) and the activity with chromogenic substrates (azocasein and elastin-orcein) was measured using the method of Knox & Kennedy (1988).

Substrate gel electrophoresis

To determine protease activity in the excretory–secretory products, samples containing 20 μg protein were run on 10% sodium dodecyl sulphate polyacrylamide gels (SDS–PAGE) containing 0.1% (w/v) gelatin (Thomas *et al.*, 1994). Electrophoretic conditions were similar to those of Laemmli (1970) but reducing agents were not used in sample buffer. Following electrophoresis the gels were washed with Triton X-100 for 1 h and incubated overnight in 0.1 M phosphate buffer pH 7.0 or 0.1 M citrate buffer pH 5.2 at 37°C. Gels were stained with Coomassie blue and destained with 25% methanol and 7.5% acetic acid until a clear zone of hydrolysis appeared in the blue background.

pH determination and inhibitor study

For pH determination, a 0.1 M citrate phosphate buffer (pH 3.0–6.0), a 0.1 M phosphate buffer (pH 7.0) and a 0.1 M glycine-NaOH buffer (pH 8.0–9.0) were used. Enzymes were characterized using different inhibitors at different concentrations with prior incubation of 30 min before starting the enzyme reaction.

Antibody inhibition

Purified collagenase was incubated with IgG separated from normal human and *W. bancrofti* infected human sera for 30 min at 37°C before starting the reaction. Collagenase activity was assayed as previously described.

Table 1. Stage-specific activity of *Setaria cervi* collagenase at pH 7.0.

Sample	Specific activity ± S.D.*
Adult female extract	2.4 ± 0.004
Microfilarial extract	6.01 ± 0.02
Excretory-secretory (ES) product of mf	41.01 ± 0.21
ES product of adult female	42.60 ± 0.23

*Mean of 10 determinations. Specific activity is defined as μg of collagen hydrolysed per mg of protein per minute at 37°C.

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Results

Table 1 shows the stage specific collagenase activity of *S. cervi*. Excretory–secretory products of microfilariae and adult females exhibited almost comparable specific activities of collagenase. Microfilarial extracts showed about 7 times less specific activity compared with excretory–secretory products of mf and adult females but about 2.5 times more specific activity in comparison with extracts of adult females. Substrate gel electrophoresis revealed two bands of hydrolysis (fig. 1).

Enzymes were purified by DEAE-sepharose CL6B and Sephadex G-100 column chromatography. Two enzyme peaks, one in an unbound fraction and another in a 0.1 M NaCl gradient, were obtained in DEAE-sepharose CL6B column chromatography. When the unbound fraction was further purified via Sephadex G-100, a single protein band was obtained using silver staining (fig. 2). The bound fraction had too little protein to proceed for further purification and showed two bands using silver staining (fig. 2). The fold purification of bound and unbound fractions were 32.5 and 84, respectively. Activity staining of the unbound fraction confirmed the protein band as a protease (fig. 3a). The protease activity of the bound fraction is associated with an upper protein band (fig. 3b). The molecular mass of the unbound fraction was found to be 50 kDa whereas that of the bound fraction was 70 kDa. The pH optimum of the 50 kDa collagenase was 5.2 and that of the 70 kDa was 7.0.

There was significant inhibition of 50 kDa collagenase with the cysteine inhibitor leupeptin and many fold augmentations with the thiol group stabilizer dithiothreitol (DTT) (table 2). Inhibitor studies of 70 kDa collagenase showed considerable inhibition with the metal chelator and serine group inhibitors as well as with the divalent cations Cu^{2+} and Zn^{2+} (table 2). There was complete inhibition of 70 kDa collagenase with 1,10-phenanthroline and di-isopropyl fluorophosphate (DIFP) (fig. 4). An antifilarial drug, diethylcarbamazine (DEC) did not show any significant inhibition of collagenase under *in vitro* conditions (table 2). Immunoglobulin subclass IgG separated from *W. bancrofti* infected human sera significantly inhibited both the collagenases under *in vitro* conditions (table 3).

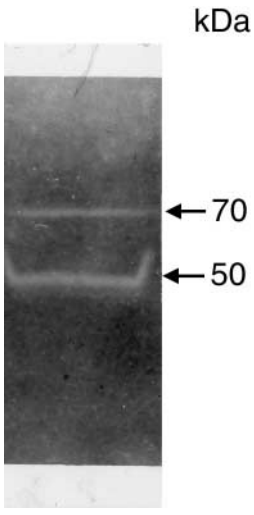

Fig. 1. Hydrolysis of gelatin by *in vitro* released proteases of *Setaria cervi* microfilariae.

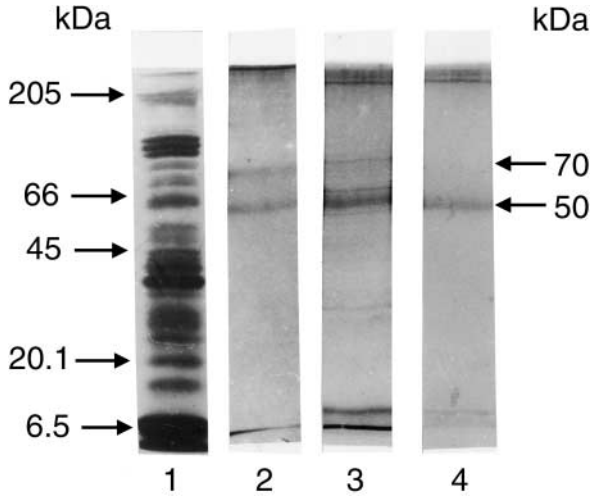

Fig. 2. Silver staining of SDS–PAGE at different purification steps of *in vitro* released proteases of *Setaria cervi* microfilariae. Lane 1, crude excretory-secretory product of *Setaria cervi* microfilariae; lane 2, bound fraction of DEAE-sepharose CL6B; eluted with 0.1 M NaCl, having collagenase activity; lane 3, unbound fraction of DEAE-sepharose CL6B having collagenase activity; lane 4, collagenase peak eluted from Sephadex G100.

Discussion

The present study indicates that filarial worms possess two different collagenases. These enzymes hydrolyse collagen type V from the bovine Achilles tendon. On the basis of per mg of protein, different stages have variations in the level of collagenase activity. The microfilarial stages show more activity when compared with adult females of *S. cervi.* The *in vitro* products from the mf show almost the same amount of collagenase activity as those of adult females. This type of stage specific activity has also been detected in filarial worms such as *O. volvulus* and *B. malayi* by Petralanda *et al.* (1986), except the latter authors reported more activity in adult female worms than in microfilarial stages. On purification using DEAE-sepharose CL6B and Sephadex G-100, two collagenses of molecular mass 50 kDa and 70 kDa were obtained. At 37°C, the *S. cervi* 50 kDa and 70 kDa collagenases were found to hydrolyse collagen optimally at pH 5.2 and 7.0, respectively. Collagenases of *Schistosoma mansoni* with pH optima of 7.5 and of *Strongyloides ratti* with pH optima in the vicinity of 7.0 have been described by Lewart & Lee (1954). A cysteine proteinase (cruzipain) from *Trypanosoma cruzi* also showed two pH optima, 5.0 and 7.0 (Cazzulo *et al.*, 1990). In addition to collagen, these enzymes also hydrolyse general protease substrates such as human haemoglobin, casein and azocasein but failed to give any activity with elastin-orcein.

Inhibitor studies in the present case revealed that

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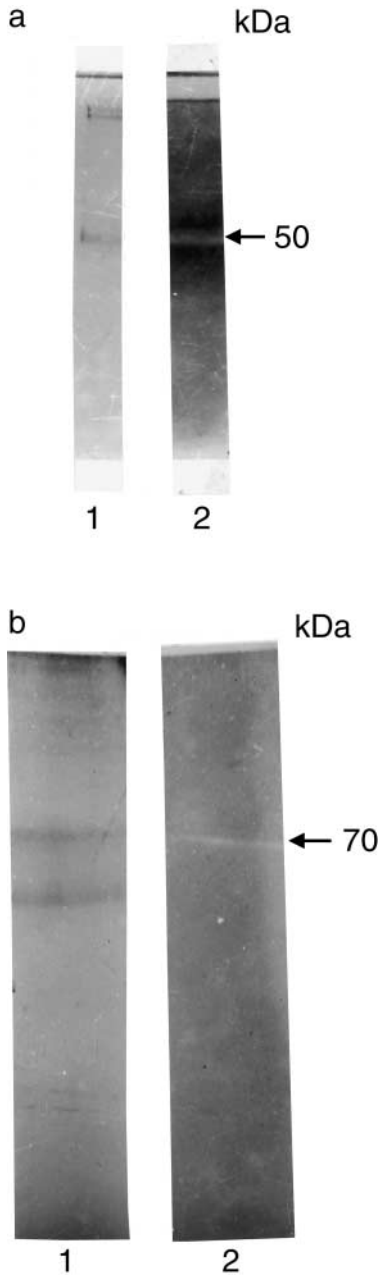

Fig. 3. Silver (lane 1) and activity (lane 2) staining of purified 50 kDa (a) and 70 kDa (b) collagenases of the microfilarial excretory–secretory product of *Setaria cervi*.

50 kDa collagenase was significantly inhibited by a cysteine group inhibitor leupetin, NEM and activity was enhanced many times by a thiol group stabilizer DTT indicating it to be a thiol protease in nature. The 70 kDa collagenase was considerably inhibited by EDTA, 1,10-phenanthroline, PMSF, DIFP and the divalent cations Cu^{2+} and Zn^{2+}. Thus, inhibition of 70 kDa collagenase by metal-chelating agents and some divalent cations suggests that this enzyme is metalloprotease akin to vertebrate morphogenic collagenases but different from

Table 2. Effect of inhibitors of 50 kDa and 70 kDa collagenases using collagen as a substrate.

Inhibitors	Concentration	% Inhibition (−)/ activation (+)	
		50 kDa	70 kDa
NEM	5 mM	−41	
Leupeptin	10 μg ml^{-1}	−94	
DTT	1 mM	+323	
PMSF	5 mM		−46
EDTA	10 mM		−74
Cu^{2+}	1 mM		−64
Zn^{2+}	1 mM		−66

NEM, *N*-ethylmaleimide; DTT, dithiothreitol; PMSF, phenyl-methylsulphonyl fluoride; EDTA, ethylenediaminetetra-acetic acid.

eukaryotic digestive collagenase that often resembles trypsin (Lecroisey *et al.*, 1979).

The present finding that antibody stimulation by infection inhibits collagenase activity is strongly supported by previous workers. Antibody generation against proteases of *Ascaris suum* and *Schistosoma mansoni* inhibits activity (Chappell & Dresden, 1988; Knox & Kennedy, 1988) and might be protective. Moreover, the ability of proteases to elicit a protective response has been demonstrated for an enzyme present in erythrocytes infected with *Babesia bovis* (Commins *et al.*, 1985). In the present study, collagenases of *Setaria cervi* were inhibited

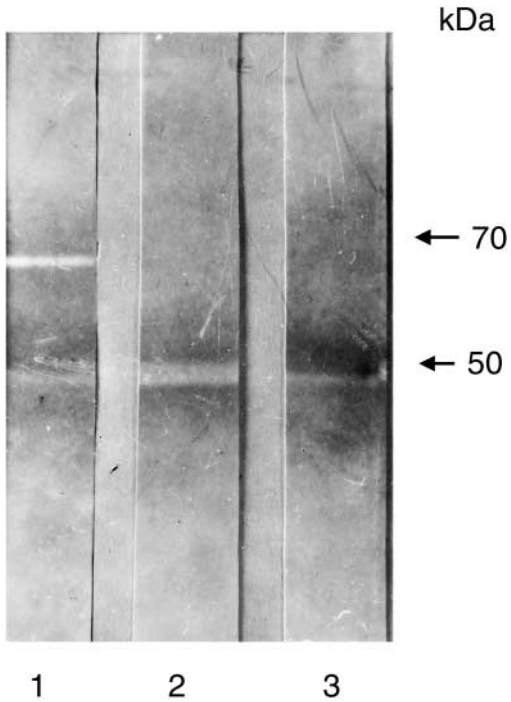

Fig. 4. Effects of inhibitors on purified 70 kDa collagenase from *in vitro* released product of *Setaria cervi* microfilariae in gel. Lane 1, control; lane 2, 1,10-phenanthroline; lane 3, di-isopropyl fluorophosphate.

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Table 3. Inhibition of 50 kDa and 70 kDa collagenases with IgG separated from *Wuchereria bancrofti* infected human sera.

Samples	Percentage inhibition*	
	50 kDa	70 kDa
Collagenase	0	0
Collagenase + normal IgG	21	10
Collagenase + filarial IgG	84	91

*Inhibition of enzyme activity by IgG separated from *W. bancrofti* infected sera, was performed as described in materials and methods.

by IgG separated from *W. bancrofti* infected human sera. The inhibition of enzymes by IgG separated from *W. bancrofti* human sera was significantly high but this was negligible compared with normal human serum IgG. This suggests that *S. cervi* collagenases may be antigenic and cross reactive with *W. bancrofti* infected sera. On this basis, it can be speculated that antigenic and enzyme active sites may be similar as antigen–antibody binding results in the loss of collagenase activities. Since collagenases of *S. cervi* mf react with protective immunoglobulin from human filarial cases they could be targeted as vaccine candidates.

A possible role for these collagenases is to create a passage for microfilariae through the host tissues. At the same time the presence of a general proteolytic activity enables this enzyme to hydrolyse other proteins that might assist the parasite in feeding and energy metabolism.

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