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Adult 175 kDa collagenase antigen of *Setaria cervi* in immunoprophylaxis against *Brugia malayi* in jirds

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Abstract

A 175 kDa antigen fraction with collagenase activity was isolated and purified from somatic extracts of adult *Setaria cervi* females using column chromatography involving consecutive steps of DEAE-Sepharose CL6B and Sephadex G-100. The optimum pH for 175 kDa collagenase was found to be pH 7.0. Sensitivities to a variety of inhibitors and activators indicated that the 175 kDa collagenolytic enzyme was metalloserine in nature. The enzyme hydrolysed a variety of protein substrates such as haemoglobin, casein, azocasein (general substrates) and collagen, FALGPA (furanoyl-acryloyl-leu-gly-pro-ala), the specific substrate of collagenase. The enzyme showed 57% inhibition by jird anti-somatic collagenase antibodies and reacted insignificantly with normal jird sera. Further analysis was undertaken on the immunoprophylactic potential of 175 kDa collagenase in inducing immunity against *Brugia malayi* (a human filarial parasite) in jirds (*Meriones unguiculatus*) *in vitro* and *in situ*. Immune sera of jirds raised against this antigen promoted partial adherence of peritoneal exudate cells to *B. malayi* microfilariae (mf) and infective larvae (L3) *in vitro* and induced partial cytotoxicity to the parasites within 48 h. The anti-*S. cervi* 175 kDa antigen serum was more effective in inducing cytotoxicity to *B. malayi* L3, than mf. In the microchambers implanted inside immune jirds, host cells could migrate and adhere to the mf and infective larvae thereby killing them partially within 48 h.

Introduction

Filariasis is a chronic debilitating disease caused by nematode parasites of the order Filaroidea. Due to the unavailability of the human filarial parasite *Wuchereria bancrofti*, the bovine filarial parasite *Setaria cervi* has been chosen for the present study. This parasite resembles the human parasite in its nocturnal periodicity and antigenic pattern. Earlier attempts have been made by many workers to identify immunoprotective antigens in other filarial parasites (Freedman *et al.*, 1989; Cheirmaraj *et al.*, 1991). The animal models *Meriones unguiculatus* and *Mastomys natalensis* for the maintenance of a closely related filarial parasite *Brugia malayi* are useful in vaccine studies against filariasis. The development of vaccines against filarial infections can act as an additional measure to existing therapeutic methods for the elimination of this disease. Proteases in several non-filarial parasites have been targeted as vaccine candidates, namely an anti-coagulant protease produced by *Ancylostoma caninum* (Hotez *et al.*, 1985), the excretory secretory proteases of tissue-stage larvae of *Ascaris suum* (Knox & Kennedy,

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1988) and the proteinases released by various ovine gastrointestinal nematodes. Also the proteases of schistosomes (Pino-Heiss *et al.*, 1986; Davis *et al.*, 1987; Verity *et al.*, 2001) and *Leishmania* have been shown to have a role in protective immune response against these parasites. The electrophoresis of *S. cervi* adult female somatic extract using sodium dodecyl sulphate (SDS) substrate gel revealed a number of proteolytic activities. As 175 kDa collagenase isolated from somatic extracts of *S. cervi* adult females was found to be inhibited by *W. bancrofti* infected sera, this collagenase was selected for studying its immunoprophylactic potential. Therefore the present communication reports on the characterization and vaccine potential of this antigen by inducing antibody-dependent cell-mediated cytotoxicity (ADCC) reaction against the filarial parasite *B. malayi* in jirds *in vitro* and *in situ*, thereby killing the microfilariae and L3 larvae of *B. malayi*.

Materials and methods

Preparation of somatic extracts

Adult females of *S. cervi* were dried, weighed and a 10% worm extract was prepared by homogenizing these parasites at 4°C in 0.1 M phosphate buffer pH 7.0 using a Remi made homogenizer type RQ 127A centrifuging at 10,000 rpm and recovering, the clear supernatant. This supernatant was further concentrated 4–5 times and dialysed against the same buffer at 4°C.

The protein content was estimated by the method of Lowry *et al.* (1951) and the proteolytic activity was assayed using the method of Singh & Rathaur (1996).

Purification and characterization of 175 kDa collagenase from adult S. cervi *females*

The 175 kDa collagenase within the supernatant was purified by a DEAE-Sepharose CL6B ion exchanger. The concentrated crude female extract was applied to the DEAE-Sepharose column (regenerated as per instruction given by the company) and washed with 0.1 M phosphate buffer pH 7.0 at a 15 ml h^{-1} flow rate. Eluates of 2.0 ml were collected. After the unbound proteins were washed out, the column was eluted with sodium chloride gradient (0.05–0.3 M). The enzyme was further purified by Sephadex G-100 column chromatography. The enzyme peak obtained from the DEAE-Sepharose was concentrated and loaded on to a Sephadex G-100 column and proteins were eluted with 0.1 M phosphate buffer pH 7.0 at the flow rate of 15 ml h^{-1} and elutes of 2.0 ml were collected. Fractions containing enzyme activity were pooled, concentrated and used for characterization.

The purified enzyme was further resolved by 10% SDS substrate gel electrophoresis (copolymerized with 0.14% w/v gelatin) as described by Thomas *et al.* (1994).

The purified enzyme was tested with different substrates such as haemoglobin, casein and collagen (1.3 mg) as described by Singh & Rathaur (2003) and using azocasein (4 μg) and elastin-orcein (1.5 mg) as described by Knox & Kennedy (1988). Furanoyl-acryloyl-leu-gly-pro-ala (FALGPA; 20 mM) was used as a specific substrate for collagenase. The enzyme was further characterized on the basis of inhibitor and activator studies. For this purpose inhibitors/activators were in cubated for 20 min at 37°C prior to the start of the enzyme assay. After incubation of enzyme with these agents enzyme activity was assayed as described above.

Immunization of jirds with filarial antigen

Polyclonal antibodies against a purified adult *S. cervi* female enzyme were raised in jirds following the procedure of Cheirmaraj *et al.* (1991). Two groups of jirds (*Meriones unguiculatus*), control and test, were immunized intraperitoneally (ip), four in each group, with purified adult *S. cervi* enzyme protein. The first dose consisted of 25 μg of antigenic protein emulsified in Freund's complete adjuvant. The second and third doses of a similar amount, emulsified in Freund's incomplete adjuvant were given at intervals of 10 days. Control jirds received only phosphate buffered saline emulsified in Freund's adjuvant as described above. A week after the last dose of antigen, jirds were bled from the retro-orbital plexus and immune sera were isolated. The antibody titre was checked by ELISA as described above.

Peritoneal exudate cells

Peritoneal exudate cells (PEC) from normal jirds were collected from the peritoneal cavity by washing with sterile medium RPMI 1640. The cells were washed with the same medium supplemented with 10% fetal calf serum (FCS) and the viability of the cells was assessed by trypan blue dye exclusion.

In vitro *cytotoxicity assay*

The cytotoxicity assay was carried out as described by Chandrashekhar *et al.* (1985a, 1990). Briefly, 100 mf or 10 L3 of *B. malayi* in 50 μl of RPMI 1640 were incubated with 50 μl of PEC (2×10^5 cells/mf or 5×10^4 cells/L3) and 50 μl of normal or immune jird serum in a 96-well culture plate (Costar Inc., Massachusetts, USA). The plate was incubated in an atmosphere of 5% CO_2 using a CO_2 incubator at 37°C. At different periods of incubation (24 and 48 h), samples were examined microscopically for cellular adherence and cytotoxicity to microfilariae and infective larvae. The percentage of cytotoxicity was expressed by considering the number of immobile or dead parasites within the experimental period. Dead parasites were those which became entangled with the peritoneal exudates cells and showed no movement.

$$\%\,\text{Cytotoxicity} = \frac{\text{No. of dead or immobile parasites}}{\text{Total no. of parasites recovered}} \times 100$$

In situ *cytotoxicity assay (micropore chamber technique)*

Micropore chambers were assembled using 14 × 2 mm plexi glass rings (Millipore Filter Corp, Bedford Massachusetts, USA) and 3 mm nucleopore polycarbonate membrane (Thomas Scientific USA) as described by Weiss & Tanner (1979). The micropore chambers were loaded with 10 L3 or 100 mf of *B. malayi* in RPMI 1640 medium via an aperture (diameter 1 mm) at the side of the

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plexi glass ring and then sealed with paraffin wax and subsequently with MF cement (Millipore Filter Corp. USA). The chambers were implanted intraperitoneally into immunized and control jirds through an incision of 2–3 cm and the skin was sutured under anaesthesia. After 24 and 48 h the chambers were taken out and the contents were removed with a Pasteur pipette and examined microscopically.

Antibody inhibition of 175 kDa enzyme

Purified enzyme (3 μg) was incubated with 1.5 μg serum protein (control and test jirds) for 45 min at 37°C with continuous shaking before starting the collagenolysis. After 45 min incubation, enzyme activity was assayed as previously described.

Results

Purification of enzyme

Adult *S. cervi* protease was purified by the combination of DEAE Sepharose CL6B and Sephadex G-100 column chromatography. Protease activity found in 0.1 M NaCl gradient in DEAE-Sepharose chromatography was further purified to homogeneity using Sephadex G-100. Purification at each step is shown on 10% SDS-PAGE along with molecular weight markers. (fig. 1). The molecular weight of the enzyme eluted in a 0.1 M NaCl gradient was 175 kDa.

Substrate specificity

The purified enzyme hydrolysed general protease substrates such as human haemoglobin, casein and collagen at optimum pH but no affinity was shown to elastin-orcein, a specific substrate for elastase. The maximum activity was observed with FALGPA (a specific substrate for collagenase). Results of substrate specificity for partially purified protease are shown in table 1. Based on these observations enzyme was characterized using collagen type V as substrate.

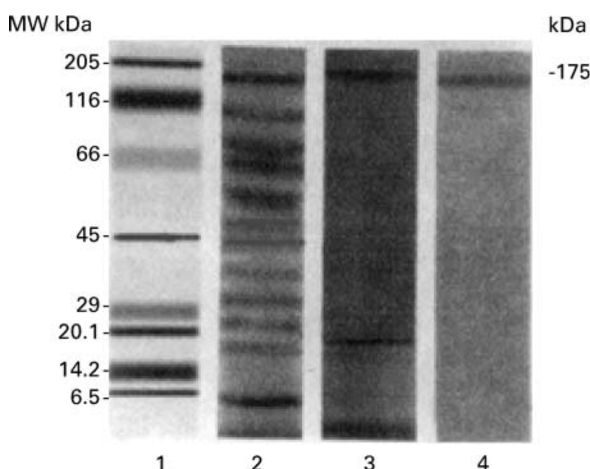

Fig. 1. Ten per cent SDS-PAGE of adult *Setaria cervi* collagenase of different purification steps. Lane 1, coloured molecular weight marker proteins; lane 2, silver staining of 10% SDS PAGE of crude somatic extract of adult *S. cervi*; lane 3, silver staining of bound fraction of DEAE Sepharose CL6B, eluted with 0.1 M NaCl; lane 4, bound fraction after purification by Sephadex G-100.

Effect of inhibitors and activators

Table 2 shows the effect of inhibitors and activators on the activity of the 175 kDa enzyme at pH 7.0. At this pH 175 kDa collagenase showed sensitivity towards a metal chelator, a serine group inhibitor and divalent cations. The residual activity obtained by leupeptin at the concentration of 0.2 mg ml^{-1} was 26.32 ± 0.04 units ml^{-1} whereas the augmented enzyme activity obtained by 5 mM dithiothreitol (DDT) was 89.6 ± 0.02 units ml^{-1}. The metal chelator by ethylenediaminetetra-acetic acid (EDTA) at 10 mM concentration showed 22.4 ± 0.03 units ml^{-1} residual activity. The Zn^{++} ions at 2 mM concentration showed 23.8 ± 0.04 units ml^{-1} residual activity, and the residual activity obtained by the serine group inhibitor phenyl-methylsulphonyl fluoride (PMSF) was 16.8 ± 0.02 units ml^{-1}. The thiol group inhibitor cystatin at 0.004 mg ml^{-1} concentration showed 33.6 ± 0.03 units ml^{-1} residual activity, whereas Ca^{++} ions exhibited 95.2 ± 0.03 units ml^{-1} residual activity.

Table 3 presents a summary of the inhibition of 175 kDa collagenase activity by specific antibodies raised against this enzyme. The residual activity obtained by jird antisomatic collagenase antibodies was 24.08 ± 0.03 compared with 48.62 ± 0.02 units ml^{-1} in control immune jird serum.

Immunoprophylactic potential of purified adult S. cervi *175 kDa collagenase against filarial parasite* B. malayi

The *in vitro* cytotoxic assays showed that the immune sera of jirds raised against adult *S. cervi* 175 kDa collagenase promoted the adherence of PEC to *B. malayi* microfilariae and infective larvae and induced partial cytotoxicity to the parasites within 24–48 h (table 4). The anti-adult *S. cervi* 175 kDa collagenase antibodies were more effective in killing *B. malayi* L3 than *B. malayi* mf. However both control and test jird antisera had different cytotoxic effects on mf and L3 stages of *B. malayi*. The cytotoxicity in the immunized jirds varied from

Table 1. Substrate specificity of 175 kDa collagenase in adult *Setaria cervi*.

Substrate	Activity* (units ml^{-1} min^{-1})
General	
Haemoglobin	40.0 ± 0.04
Casein	41.6 ± 0.03
Azocasein	48.5 ± 0.02
Specific	
Elastin orcein	0.0
Collagen (type V)	54.9 ± 0.05
FALGPA	56.0 ± 0.07

Values are the mean ± SD of 10 determinations; *one unit of enzyme activity is the amount of enzyme needed to produce 1 μg of product per ml per min under experimental conditions, i.e. at 37 °C.

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Table 2. Effect of inhibitors/activators on pure adult *Setaria cervi* 175 kDa collagenase activity at pH 7.0.

Inhibitor/activator	Final concentration	Control activity (units ml^{-1})	Residual activity (units ml^{-1})	Enhanced activity (units ml^{-1})
EDTA	10 mM	56.0 ± 0.02	22.4 ± 0.03	–
PMSF	5 mM	56.0 ± 0.03	16.8 ± 0.02	–
Leupeptin	0.2 mg ml^{-1}	56.0 ± 0.04	26.32 ± 0.04	–
Cystatin	0.004 mg ml^{-1}	56.0 ± 0.03	33.6 ± 0.03	–
D.T.T.	5 mM	56.0 ± 0.02	–	89.6 ± 0.02
Zn^{2+}	2 mM	56.0 ± 0.04	23.8 ± 0.04	–
Ca^{2+}	2 mM	56.0 ± 0.03	–	95.2 ± 0.03

Values are the mean ± SD of 10 determinations; common controls were run for all assays; enzyme activity was assayed using FALGPA as substrate.

12 to 21% in 24 h and 32 to 40% in 48 h whereas the cytotoxicity in control jirds was almost negligible, i.e. 3% in 24 h and varied between 4 to 5% in 48 h.

Microscopic observations of the micropore chambers implanted in jirds immunized with 175 kDa collagenase of adult *S. cervi* female showed some migration of host lymphocytes, polymorphonuclear cells and a few macrophages into the chambers leading to their adherence and partial killing of microfilariae or infective larvae within 24–48 h as shown in table 5.

Cytotoxicity in immunized jirds varied from 10 to 15% in 24 h and 35 to 42% in 48 h. On the other hand, in the chambers implanted in control jirds the cytotoxicity to the parasites was not observed in 24 h but was just 4 to 6% in 48 h.

Discussion

A significant amount of collagenase activity in various life stages of *B. malayi*, *Onchocerca volvulus*, *Dirofilaria immitis* and *Ascaris lumbricoides* (Petralanda *et al.*, 1986) has been reported.

Collagenase from the somatic extract of adult *S. cervi* was purified by two consecutive steps of DEAE-Sepharose CL6B and Sephadex G-100 column chromatography. Inhibitor and activator studies showed that the 175 kDa enzyme is of the metalloserine type. Likewise, metalloserine collagenase has been reported in IVR products of *S. cervi* mf by Singh & Rathaur (2003).

From the outset on immunological studies of nematodes, a major objective has been the development of a protective immunity stimulating vaccine. Studies on the long term effect on the development of infective larvae to adult worms and information on related immune responses are useful in assessing the potential use of such antigens as vaccines aimed at preventing infection (Phillip *et al.*, 1988).

Jird polyclonal antibodies raised against adult *S. cervi* 175 kDa collagenase were used to assess their inhibitory effect on the enzyme. The anti-collagenase antibodies were found to inhibit enzyme activity up to 57% at pH 7.0. Earlier, Singh & Rathaur (2003) have reported that IgG separated from *W. bancrofti* infected serum has shown significant inhibition of collagenases isolated from *in vitro* released product of *S. cervi* mf.

Immunoprophylaxis may serve as an additional adjunct along with chemotherapy and antilarval measures for successful filarial control. Several types of crude and purified filarial antigens have been explored as protective immunogens. These include adult or larval extract antigens on the parasites surface, ES products and functional enzyme molecules. Therefore a further study was undertaken up to explore the possibility of using adult *S. cervi* 175 kDa collagenase as a vaccine candidate

Table 3. Inhibition of adult *Setaria cervi* female 175 kDa collagenase with jird anti-somatic collagenase antibodies.

Samples	Control activity (units ml^{-1})	Residual activity (units ml^{-1})
Collagenase	54.0 ± 0.3	–
Collagenase + normal jird serum	55.0 ± 0.1	48.62 ± 0.2
Collagenase + test jird serum	56.0 ± 0.2	24.08 ± 0.3

Values are mean ± SD of 10 different determinations; 30 μg protein from control and immune jird sera were incubated with 3 μg pure collagenase and assayed for residual activity.

Table 4. Serum dependent cellular cytotoxicity to *Brugia malayi* parasites *in vitro* by jird anti-*Setaria cervi* 175 kDa collagenase.

Jird serum	% Cytotoxicity 24 h		% Cytotoxicity 48 h	
	Mf	L3	Mf	L3
Control	3 ± 3	–	4 ± 7	5 ± 11
Anti-adult *S. cervi* collagenase*	12 ± 4	21 ± 8	32 ± 10	40 ± 15

Mf, microfilariae: L3, third stage larvae, *sera collected from four jirds and tested separately; values are mean ± SD.

Table 5. Cytotoxicity to *Brugia malayi* parasites in micropore chambers implanted into jirds immunized with adult *Setaria cervi* collagenase.

Jird	% Cytotoxicity 24 h		% Cytotoxicity 48 h	
	Mf	L3	Mf	L3
Control	–	–	4 ± 7	6 ± 8
Immunized group	10 ± 4	15 ± 6	35 ± 7	42 ± 12

Mf, microfilariae; L3 third stage larvae; values are mean ±SD four jirds per group.

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by evaluating its role in inducing antibody dependent cell mediated cytotoxicity (ADCC) reaction both *in vitro* and *in situ* assays for killing the microfilariae and L3 larvae of *B. malayi*.

The observations in *in vitro* ADCC reactions showed that the antibodies raised against 175 kDa collagenase of adult *S. cervi* females induced 32% and 40% cytotoxicity after 48 h to the *B. malayi* mf and L3 stages respectively. This, to our knowledge, is the first observation in case of *S. cervi* for inducing partial immunity in the fully permissive host (i.e. jirds).

The non-living parasites or their extracts have generally failed to confer resistance in fully permissive hosts, although they may do so in animals that are semi-permissive for parasite development (Mehta *et al.*, 1981a; Carlow & Philipp, 1987). Both antibody and complement mediated effector mechanisms have been shown to operate on microfilariae and infective larvae *in vitro* (Subrahmanyam *et al.*, 1976; Tanner & Weiss, 1978; Haque *et al.*, 1981; Mehta *et al.*, 1981b; Sim *et al.*, 1982; Chandrashekhar *et al.*, 1985a,b, 1990). The nematode epicuticle may act as substrate for antibody and complement mediated adherence of host cells that may result in damage to or death of the parasite (Capron *et al.*, 1982). Neutrophils, macrophages and eosinophils are all known to possess surface receptors for the Fc part of IgG (Rabellino & Metcalf, 1975; Wong & Wilson, 1975). The predominant isotope responsible for the ADCC reaction was found to be IgG (Chandrashekhar *et al.*, 1985a,b; World Health Organization, 1987).

The micropore chamber technique was also employed to check whether a similar ADCC reaction can occur *in situ*. These chambers implanted in animals are advantageous since they provide a closer physiological environment than *in vitro* cultures for larval growth and survival and thus for assessing the host effector mechanism (Weiss & Tanner, 1979; Rajasekariah *et al.*, 1989). In the present study, microfilariae and infective larvae were attacked to some extent by host cells which migrated into the chambers implanted in immunized jirds within 24–48 h. *Setaria cervi* collagenase induced almost 35% and 42% cytotoxicity after 48 h to the *B. malayi* mf and L3 stages respectively. It seems that cytotoxicity might have been further enhanced on increasing the immunization time.

In earlier studies Chandrashekhar *et al.* (1990) showed that *B. malayi* L3 were attacked by the host macrophages and polymorphs in microchambers from 16–24 h after implantation in immunized rats. However, Abraham *et al.* (1986) observed that it took 10 days for the host cells to kill *Dipetalonema viteae* L3 in diffusion chambers implanted in infected jirds, suggesting a necessary developmental change in the larvae for generating susceptibility to immune attack. These variations in cytotoxicity reactions under *in situ* conditions suggest that the responsible factors seem to be dependent on parasite species and the host (permissive and non-permissive). The involvement of macrophages in mediating cytotoxicity reactions has also been demonstrated against other filarial parasites, namely *Dipetalonema setariosum* adults (Worms & Mclaren, 1982) and *Litomosoides carinii* microfilariae (Nelson *et al.*, 1976).

Besides filarial proteases, proteinases (cathepsin L1 and cathepsin L2) secreted by liver flukes (*Fasciola hepatica*) showed immunoprotection when used as vaccine candidates in cattle (Dalton *et al.*, 1996). Mulcahy & Dalton (2001) have suggested that these vaccines exhibit high anti-embryonation/anti-fecundity effects on *F. hepatica* worms that survived in vaccinated cattle and can have a major impact on the transmission of *F. hepatica* to its intermediate host. An anticoagulant metalloprotease produced by adult *Ancylostoma caninum* (Hotez *et al.*, 1985) has been shown to be an important target for vaccine development. The *in vitro* released proteases of the tissue stage larvae of the nematode *Ascaris suum* have been shown to be highly antigenic and the antibody obtained from infected animals can inhibit the parasite's protease (Knox & Kennedy, 1988). Even the proteinases released by various gastrointestinal nematodes have previously been shown to be highly antigenic and targets of protective immunity. The role of proteinases in inducing protective immune response has also been demonstrated in schistosomes (Davis *et al.*, 1987; Pino-Heiss *et al.*, 1986; Verity *et al.*, 2001). The role of proteases in immunoprotection has also been shown in case of *Leishmania* (Thomas *et al.*, 1994).

Thus it can be speculated that as more information is being generated on the immunity to filarial and non-filarial infections, varying strategies employed can help to identify proteases as protective antigens in these infections. It seems that proteases can have a role in protective immunity against several parasitic diseases and the present results suggest a possible role for *S. cervi* proteases in immunoprotection by antibody-dependent cell-mediated cytotoxicity.

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