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PROTEOLYTIC ENZYMES IN EXTRACTS OF *SCHISTOSOMA MANSONI* CERCARIAE

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SUMMARY

The enzymatic mechanisms by which cercariae of *Schistosoma mansoni* invade mammalian skin have been investigated. Cercarial extracts prepared by sonication were assayed for activity against a variety of substrates including purified components of connective tissues. These extracts contain activity against gelatin, casein, Azocoll and elastin, but do not hydrolyze undenatured soluble or fibrous collagen. Cercarial extracts are very active against a chondromucoprotein complex from cartilage but do not hydrolyze the major mucopolysaccharide component of this complex. Sephadex G-150 chromatography of cercarial extracts revealed the existence of multiple peaks of proteolytic activity. It is suggested that cercarial proteases exert their main activity against the protein backbone of the protein–polysaccharide present in mammalian connective tissues.

INTRODUCTION

The initial phase of infection of susceptible mammals by the trematode parasite *Schistosoma mansoni* involves the penetration of their skin by the cercarial stage of this organism and its migration through the dermis. The notion that these processes might occur by the secretion of lytic enzymes has been entertained for some time. Two approaches to verify this notion have been utilized. Utilizing light microscopy Lewert and Lee[1] demonstrated extensive alterations of the protein–polysaccharide ground substance of the dermis and the epidermal basement membrane. Bruce *et al.*[2] and Rifkin[3] have recently studied, by electron microscopic techniques, the changes in skin morphology during penetration and migration and concluded that cercariae utilize a heterogeneous complex of lytic enzymes for these processes.

Other investigators have attempted to analyze for the presence of proteolytic enzymes in cercarial extracts. Thus, Lewert and Lee[4] reported the presence of "collagenase-like" enzymes; while Milleman and Thonard[5] were unable to detect collagenolytic activity. Stirewalt and Fregeau[6] studied hemoglobin-degrading

activities. Gazzinelli *et al.*[7] have carried out the most extensive investigation of cercarial proteases using DEAE-Sephadex chromatography and demonstrated the presence of multiple proteolytic activities against both casein and elastin.

This investigation was initiated to reinvestigate the presence or absence of specific collagenolytic enzymes in cercarial extracts and to define the natural substrates of these cercarial proteases by testing their activity against a variety of purified substrates representing components of mammalian connective tissues.

MATERIALS AND METHODS

Chemicals

Hyaluronic acid, chondroitin sulfate A, chondroitin sulfate B (dermatan sulfate), chondroitin sulfate C, chondroitin sulfate D, soybean trypsin inhibitor and phenylmethanesulfonylfluoride were purchased from Sigma Chemical Co. Elastin-orcein was purchased from Worthington Biochemical Corp. and Azocoll from Calbiochem. Casein was from Difco.

Guinea pig skin collagens, both unlabeled and labeled *in vivo* with [^{14}C]glycine, were isolated by the methods of Gross[8]. Autoclaved elastin, prepared by the method of Partridge *et al.*[9], and the chondromucoprotein, PP-L, prepared from bovine nasal cartilage by the methods of Gerber *et al.*[10] were gifts from Dr Nicola M. DiFerrante, Baylor College of Medicine.

Experimental

Schistosoma mansoni cercariae (Puerto Rican strain), obtained from infected snails *(Biomphalaria glabrata)*, were collected by filtration through an 8 μm Millipore filter and suspended in 0.05 M glycine–NaOH buffer, pH 8.8, to yield 30 000 to 60 000 cercariae per ml. This buffer was chosen since maximal proteolytic activity against Azocoll was obtained at this pH (Fig. 1).

Cercarial extracts were prepared by sonication (a total of three 1-min pulses) in a Bronwill Biosonik III at maximum frequency with a microprobe attachment at 0 °C. Disruption of cercarial structure was essentially complete under these condi-

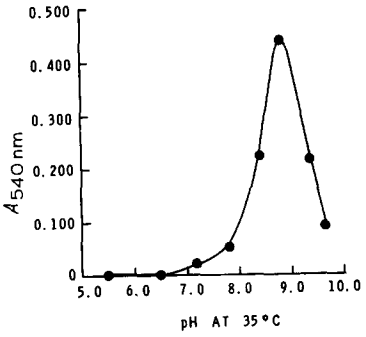

Fig. 1. pH dependence of crude cercarial proteases. Aliquots of crude cercarial proteases (50 μl) were incubated at 35 °C for 20 h with 2 mg of Azocoll in the presence of 1.5 ml of different buffer solutions (0.05 M Tris–maleate for pH 5.5 to 7.8, and 0.05 M glycine–NaOH for pH 8.25 to 9.65). Incubation was terminated by centrifugation at 15 000 × *g* for 10 min and the absorbance of the resultant supernatant determined at 540 nm in a Gilford Model 2400 spectrophotometer. Controls without cercarial extract were run in parallel.

tions, as determined by microscopic examination. Debris was removed by centrifugation at 15 000 × *g* for 10 min at 0 °C, and the resultant supernatant was dialyzed against glycine–NaOH buffer, pH 8.8, and stored at −20 °C. Enzymatic activity was retained for at least four months. These extracts contained 0.25–0.48 mg/ml protein using bovine serum albumin as a standard[11].

Molecular sieve chromatography

Sephadex G-150 chromatography was carried out at 6 °C using 0.05 M glycine–NaOH, pH 8.8, as the eluting buffer. Column dimensions were 45 cm × 2.5 cm and a flow rate of 12 ml/h was maintained. 3-ml fractions were collected and assayed for protein content at 280 nm and for enzymatic activities.

Enzymatic activities

Proteolytic activity against casein (5 mg/ml) and Azocoll (2 mg/ml) was measured spectrophotometrically after incubation at 35 °C in 0.05 M glycine–NaOH buffer, pH 8.8. Undigested protein was removed by acid precipitation (for casein) and centrifugation (for Azocoll) and the supernatants were analyzed at 280 nm (for casein) and 540 nm (for Azocoll). Proteolytic activity against gelatin (1 mg/ml) was assayed at 35 °C by determining solubilization of radioactivity from [^{14}C]glycine-labeled heat-denatured guinea pig skin collagen after precipitation of undigested gelatin by trichloroacetic acid–sodium tungstate[12].

The presence of collagenases was assayed for using both native reconstituted collagen fibers and soluble collagen. Activity on collagen fibers was measured by solubilization of radioactivity from [^{14}C]glycine-labeled guinea pig skin collagen[13] at 35 °C. Activity on acid-extracted soluble collagen was measured by determining the effect on the viscosity of these collagen solutions (1 mg/ml) after addition of cercarial extracts.

Elastolytic activity was measured at 35 °C using elastin-orcein (5 mg/ml) and autoclaved elastin (5 mg/ml) by the methods of Gazzinelli *et al.*[7].

Activity against purified acid mucopolysaccharides and chondromucoprotein (PP-L) was measured by incubation of cercarial extracts with the appropriate substrates in Cannon-Manning Semi-Micro Ostwald viscometers in 0.05 M glycine–NaOH, pH 8.8 at 24 °C and 35 °C. Chondroitin sulfates A, C and D were present at 10 mg/ml while dermatan sulfate and PP-L were present at 5 and 2 mg/ml, respectively. Activity of cercarial extracts was quantitated by plotting $[\eta_{sp}]$ against incubation time and determining the initial rate of loss of viscosity.

RESULTS

Substrate specificity

The activity of extracts from *Schistosoma mansoni* cercariae against a variety of substrates is shown in Table I. Activity of the extracts was defined as hydrolysis of the substrate above that of controls containing no extract and was proportional to incubation time and to concentration over a 5-fold range of added extract. General proteolytic substrates such as casein, Azocoll (azo dye-complexed hide powder) and gelatin, as well as elastin and chondromucoprotein were hydrolyzed after incubation with cercarial extracts for 4–8 h at 35 °C in glycine–NaOH buffer, pH 8.8. On the

TABLE I

SPECIFICITY OF CRUDE CERCARIAL EXTRACTS

Substrates hydrolyzed by Cercarial extracts

Azocoll
Casein
Gelatin
Elastin
Chondromucoprotein (PP-L)

Substrates not hydrolyzed by cercarial extracts

Fibrous collagen
Soluble collagen
Hyaluronic acid
Chondroitin sulfate A
Dermatan sulfate
Chondroitin sulfate C
Chondroitin sulfate D

*Inhibitors**	*Inhibition*
Soybean trypsin inhibitor	± inhibition (10–20%)
Phenylmethanesulfonylfluoride	significant inhibition (50–90%)
Disodium EDTA	no inhibition (<10%)
Pooled human serum	complete inhibition (> 90%)

* Inhibitors were tested in the hydrolysis of Azocoll. See Materials and Methods for assay techniques.

other hand, undenatured collagen (both soluble collagen and reconstituted fibrillar collagen) as well as purified acid mucopolysaccharides were not degraded during incubation periods of 24–48 h at 35 °C.

The relative activity of these extracts against various substrates is shown in Table II. When compared to crystalline trypsin (EC 3.4.4.4), cercarial extracts were 10-fold more active against chondromucoprotein than against any other substrate.

Effect of inhibitors

The effects of a variety of protease inhibitors on cercarial proteases are shown in Table I.

Activity of cercarial extracts against Azocoll and PP-L, using the assay conditions described above, was significantly inhibited by addition of phenylmethanesulfonylfluoride (1 mM), but not by soybean trypsin inhibitor (0.01 mg/ml). The

TABLE II

ACTIVITY OF CERCARIAL EXTRACTS AGAINST VARIOUS SUBSTRATES

Activity is expressed as the amount of cercarial protein (in μg) required to yield the same rate of hydrolysis of the substrate as 1 μg of crystalline trypsin assayed under identical conditions. See Materials and Methods for assay techniques.

Substrate	*Activity*
Azocoll	58
Casein	40
Gelatin	42
Chondromucoprotein	4.5

chelating agent, EDTA (5 mM), an inhibitor of most collagenases[14], did not inhibit Azocoll-degrading activity.

Pooled human serum (0.1 ml per ml of reaction mixture) completely inhibited the Azocoll-degrading activity of cercarial extracts. In addition, individual Sephadex column fractions (see below) exhibiting proteolytic activity were also completely inhibited by serum.

Column chromatography

Greater than 90% of the Azocoll-active proteolytic activity was recovered by $(NH_4)_2SO_4$ fractionation between 30 and 70% saturation, in agreement with the data of Gazzinelli *et al.*[7] who found 70% of the caseinolytic activity between 40 and 60% saturation.

In an effort to determine the nature of the proteolytic enzymes in cercarial extracts $(NH_4)_2SO_4$ concentrated extracts and unfractionated, dialyzed crude sonicates were subjected to Sephadex G-150 chromatography. Column fractions were assayed for proteolytic activity against Azocoll, casein and gelatin substrates. The results of assays using these substrates are shown in Fig. 2, and indicate the presence of multiple activities in cercarial extracts.

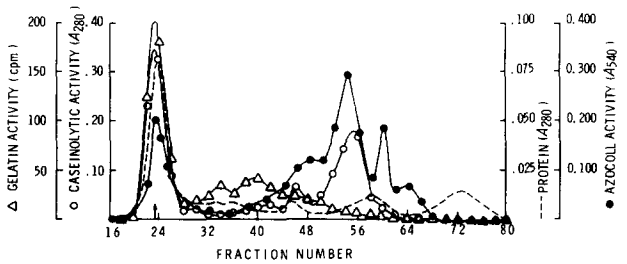

Fig. 2. Sephadex G-150 chromatography of crude cercarial proteases. Sephadex G-150 chromatography of 2.5 ml $(NH_4)_2SO_4$ concentrated cercarial extract was carried out at 6 °C with 0.05 M glycine–NaOH, pH 8.8 as the eluting buffer. 3-ml fractions were collected and analyzed for protein content by absorbance at 280 nm (– – –) and enzymatic activities (———) at 35 °C using 0.5 ml aliquots; ○—○, caseinolytic activity (A_{280}); ●—●, azocoll-degrading activity (A_{540}); △—△, gelatin-degrading activity (cpm). Arrow at Fraction 23 indicates the position of the void volume for this column (45 cm × 2.5 cm).

DISCUSSION

The results presented in this report indicate the presence of multiple proteolytic activities in extracts of *Schistosoma mansoni* cercariae. Gazzinelli *et al.*[7] using DEAE-Sephadex chromatography demonstrated the presence of one major and two minor peaks of proteolytic activity with a casein substrate, while we have found, using Sephadex G-150, approximately five peaks of activity using casein, Azocoll or gelatin substrates. Further purification is required to define the number of proteolytic species present in extracts. It is clear that the individual column fractions display quantitatively and probably qualitatively different activities against the various substrates, suggesting that they may have different substrate specificities *in vivo*.

In an effort to delineate the *in vivo* substrates for cercarial lytic enzymes a variety of compounds, representing purified components of mammalian connective

tissues, were utilized. In agreement with the results of Milleman and Thonard[5], using cercarial extracts, and of Gross and Harper (unpublished observations) using cultures of living cercariae, we fail to find collagenolytic activity; moreover none of the fractions obtained by Sephadex chromatography degrade undenatured collagen. Cercarial extracts rapidly degrade a protein-polysaccharide complex from bovine nasal cartilage (Table II) yet do not hydrolyze the major polysaccharide component, chondroitin sulfate A (ref. 15), of this complex, suggesting that these extracts act against the protein backbone of these complexes rather than the carbohydrate side chains.

These experiments indicate that specific collagenolytic enzymes are not present in cercarial extracts. The electron microscopic studies of Bruce *et al.*[2], however, suggest that some depolymerization of collagen fibers does occur during schistosomal invasion. Passage of these organisms through the fibrous network of the dermis may be facilitated by non-specific proteolytic attack on the organization of the collagen fibers. Alternatively, the depolymerization of the dermal protein–polysaccharide matrix by cercarial proteases may disrupt the structure of the fibrous network sufficiently so as to allow passage of the organisms.

The effects of various protease inhibitors indicate that the main proteolytic activity in cercarial extracts is a serine-protease. Gazzinelli *et al.*[7] initially concluded that this activity was chymotrypsin-like, but recent results by these investigators (Gazzinelli *et al.*[17]) indicate that the main proteolytic activity of cercarial extracts is not inhibited by (L-(1-tosylamide-2-phenylethyl)chloromethylketone) a specific inhibitor of chymotrypsin (EC 3.4.4.5). The inhibitory effect of pooled human serum is not surprising in view of the multiple protease inhibitors present in sera[16]. The possible presence of similar inhibitors in extracts of dermis is currently being tested.

Table II indicates that of the four substrates tested, cercarial extracts are most active against chondromucoprotein (*i.e.*, less cercarial extract is required to obtain equal rates of hydrolysis as 1 μg of trypsin). It must be noted, however, that the comparison between cercarial extracts and trypsin is not valid for elastin since this substrate is essentially impervious to trypsin. Histological studies with skin preparations are required to decide whether hydrolysis of elastin *in vitro* is accompanied by degradation of elastic fibers *in vivo*.

The data obtained thus far and the microscopic studies on infected tissues reported by Lewert and Lee[1] suggest that the major activity of proteolytic enzymes in cercarial extracts is against the protein backbone of the protein-polysaccharide ground substance of mammalian skin. Further experiments to test this suggestion using individual purified cercarial proteases and secretions of living cercariae are in progress.

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