Partial purification and characterization of an inhibitor from newborn-rat epidermis with activity against the proteinase of *Schistosoma mansoni* cercariae

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The penetration of cercariae through the skin initiates infection of the host with the human trematode parasite *Schistosoma mansoni*. Many larvae fail to migrate into the living epidermal cell layer. In order to determine if chemical as well as mechanical barriers to cercarial skin penetration exist, inhibitory activity of epidermal-cell extracts against the proteinase obtained from cercarial secretions was assayed. An inhibitor was purified 50-fold by gel filtration on Sephadex G-75 and cation-exchange chromatography at pH 5.8 and 4.9. The inhibitor has a relative molecular mass (M_r) of approx. 40 000–53 000. Oxidation of the inhibitor with *N*-chlorosuccinimide eliminated its inhibitory activity and thus indicated a critical methionine residue. The inhibitor was active against a wide spectrum of serine proteinases: porcine pancreatic elastase, human granulocyte elastase, bovine trypsin, and bovine α -chymotrypsin. However, no inhibition was detected against papain or clostridial collagenase. The inhibitor did not cross-react with antiserum to human or rat serum α_1 -proteinase inhibitor.

The penetration of cercariae through the skin initiates infection by the human trematode parasite *Schistosoma mansoni*. Skin invasion and subsequent larval migration are accomplished by both mechanical and enzymic alteration of macromolecular components of skin (Stirewalt, 1974). Biochemical studies have shown that enzymes contained in extracts of whole cercariae or isolated from cercarial preacetabular-gland secretions can degrade a variety of synthetic or natural substrates, including Azocoll and gelatin (Lewert & Lee, 1954; Stirewalt & Austin, 1973), casein (Gazzinelli *et al.*, 1966; Dresden & Asch, 1972), collagen (McKerrow & Jeong, 1982), elastin (Gazzinelli *et al.*, 1966; Dresden & Asch, 1972; Werb *et al.*, 1982), keratin (Gazzinelli *et al.*, 1966; Dresden & Lewis, 1977), proteoglycan (Landsperger *et al.*, 1982) and macromolecular components of extracellular matrix (McKerrow *et al.*, 1982). Nevertheless, a fairly high percentage of cercariae experimentally introduced on to the skin of mice and rats fails to enter the hosts (Lincicome *et al.*, 1963; Smithers & Terry, 1965). Many schistosomules die within the epidermis, the most

superficial tissue compartment (Clegg & Smithers, 1968). Penetration of the entire epidermis appears to require concerted effort by large numbers of larvae (Bruce *et al.*, 1970).

We hypothesize that epidermal cells may act as a chemical as well as a mechanical barrier to infection by *S. mansoni*. In the present study, inhibitory activity of rat epidermal-cell extracts against the secreted proteinase of cercariae was identified, and an epidermal proteinase inhibitor was partially purified and biochemically characterized.

Experimental procedures

Materials

The following materials were purchased: Azocoll, Bz-Arg-Nap·HCl, bovine α -chymotrypsin clostridial collagenase, porcine pancreatic elastase, α_1 -PI and papain (Sigma Chemical Co., St. Louis, MO, U.S.A.); bovine trypsin [1-chloro-4-phenyl-3-L-tosylamidobutan-2-one ('TPCK')-treated; Worthington Biochemical Corp., Freehold, NJ, U.S.A.]; elastin, <400 mesh, type E60 (Elastin Products Co., Pacific, MO, U.S.A.); NaB³H₄, 242 mCi/mmol (New England Nuclear Corp., Boston, MA, U.S.A.); *N*-chlorosuccinimide (Aldrich Chemical Co., Milwaukee, WI, U.S.A.); and Sprague–Dawley rats

Abbreviations used: Bz-Arg-Nap·HCl, α -*N*-benzoyl-DL-arginine-2-naphthylamide hydrochloride; PMSF, phenylmethanesulphonyl fluoride; α_1 -PI, α_1 -proteinase inhibitor; SDS, sodium dodecyl sulphate.

(Charles River Co., Wellington, MA, U.S.A.). Human granulocyte elastase was a gift from Dr. Robert Sandhaus and Dr. Zena Werb (Laboratory of Radiobiology, University of California, San Francisco, CA, U.S.A.). Two different samples of rabbit anti-(rat α_1 -PI) antisera were gifts from Dr. Aaron Janoff (Department of Pathology, State University of New York, Stony Brook, NY, U.S.A.), and Dr. Brian Kingman and Dr. Robert Webber (Lawrence Berkeley Laboratories, Berkeley, CA, U.S.A.).

Collection of cercariae and cercarial secretions

Techniques for maintaining the *S. mansoni* life cycle have been published elsewhere (Lim & Heyneman, 1972). Cercarial preacetabular-gland secretions were collected by the method of Stirewalt (1978), dialysed, and freeze-dried. This material was used as the crude cercarial proteinase as described previously (McKerrow & Jeong, 1982). Partially purified cercarial proteinase (Werb *et al.*, 1982) was also tested.

Enzyme assays

Since elastolytic activity is thought to be a significant function of cercarial secretions *in vivo* (McKerrow *et al.*, 1982), and since Azocoll has also been used extensively as a substrate in cercarial-proteinase assays (Dresden & Asch, 1972; Landsperger *et al.*, 1982), both elastin and Azocoll were used as substrates in assays of cercarial-proteinase activity and in subsequent inhibition assays.

Elastin from ox ligamentum nuchae was radioactively labelled with NaB³H₄ (Banda & Werb, 1981). The specific radioactivity of labelled elastin used in these experiments was 5×10^5 d.p.m./mg. Elastase activity was measured by determining the amount of radioactivity released after incubation of enzyme preparations at 37°C for 6–20 h with 200 µg of [³H]elastin in 100 mM-glycine/NaOH buffer, pH 9.0, containing 2 mM-CaCl₂. The final reaction volume was 300 µl. The undigested elastin was removed by centrifugation for 3 min in a Beckman Microfuge. The radioactivity remaining in 100 µl of supernatant was measured by liquid-scintillation spectrometry (Banda & Werb, 1981).

Azocolytic activity was measured by a modification of the assay used previously to identify proteolytic activity in cercarial homogenates (Dresden & Asch, 1972). Enzyme preparations were incubated at 37°C with 5 mg of Azocoll in 1 ml of 0.1 M-glycine/NaOH, pH 9.0, containing 2 mM-CaCl₂. After incubation for 0.5–3 h, the undigested Azocoll was removed by centrifugation for 3 min in a Beckman Microfuge. Degraded substrate was measured in the supernatant by spectrophotometry at 540 nm.

Porcine pancreatic and human granulocyte elastases were assayed with [³H]elastin as described above. Bovine pancreatic trypsin, α -chymotrypsin, and clostridial collagenase were assayed using Azocoll as described above.

Papain was assayed by adding enzyme preparations and 0.8 mg of Bz-Arg-Nap·HCl to 0.82 ml of 0.25 M-phosphate buffer, pH 7.5, containing 1 mM-EDTA and 0.5 mM-dithiothreitol. The mixture was incubated for 10 min at 37°C, and the product was measured by spectrophotometry at 540 nm (Barrett, 1977).

Purification of a cercarial-proteinase inhibitor from rat epidermis

The skin was removed from 2-day-old Sprague-Dawley rats, and the epidermis was separated from the dermis by incubation for 20 min in 0.24 M-NH₄Cl, pH 9.5. The epidermis was stored at –70°C until use. Pooled epidermis from about 200 rats was washed with 100 ml of 0.01 M-Tris/HCl, pH 8.0. The pellet obtained after centrifugation at 15 000 g for 20 min was homogenized with a glass homogenizer in 120 ml of 0.1 M-Tris/HCl, pH 8.0, containing 0.14 M-NaCl. Protein was extracted by stirring for 2 h at 4°C. All three solutions mentioned above contained 10 µg of PMSF/ml. The extraction was repeated, and the two supernatants were pooled, dialysed against water, and freeze-dried. The freeze-dried powder was redissolved in 9 ml of 0.02 M-Tris/HCl, pH 8.0, containing 0.14 M-NaCl and then centrifuged at 15 000 g for 20 min. The supernatant was subjected to Sephadex G-75 column chromatography (2.5 cm × 90 cm), the same buffer being used as eluent. Fractions that inhibited the elastolytic activity of the cercarial enzyme were pooled, dialysed against water, freeze-dried, and then redissolved in 6 ml of 0.05 M-sodium acetate, pH 5.8. The supernatant after centrifugation was applied to a cation-exchange column (2 cm × 18 cm) of CM-cellulose CM-52 pre-equilibrated with the same buffer. The column was washed with 3 bed vol. of the starting buffer and then eluted with a linear NaCl gradient (0–0.25 M, 300 ml total volume) in the starting buffer. Fractions that demonstrated inhibitory activity were pooled, dialysed against water, freeze-dried, and dissolved in 2 ml of 0.05 M-sodium acetate, pH 4.9. Cation-exchange column chromatography was repeated for the sample, with a column (1.5 cm × 12 cm) pre-equilibrated with 0.05 M-sodium acetate buffer, pH 4.9, and a linear NaCl gradient of 0–0.2 M in 200 ml of buffer. Fractions that demonstrated inhibitory activity against the cercarial enzyme were pooled as the epidermal proteinase inhibitor.

Inhibition assays

Inhibitory activity of the epidermal inhibitor against various proteinases was measured by in-

cubating the inhibitor and enzyme in 0.01 M-Tris/HCl, pH 8.0, at 22°C for 20 min before adding them to reaction mixtures. The inhibition of enzyme activity was calculated by assuming that substrate hydrolysis by the proteinase without inhibitor was 0% inhibition and that a control assay with substrate alone was 100% inhibition.

Polyacrylamide-gel electrophoresis

A 7.5% (w/v) polyacrylamide gel and a Tris/glycine buffer system, pH 8.9, were used under non-reducing conditions to separate and identify protein bands with inhibitor activity (Davis, 1964).

An SDS/9.0% polyacrylamide gel was used to calculate approximate M_r values for the major protein species. Protein samples were first heated in a boiling-water bath for 5 min in the presence of SDS and mercaptoethanol (Weber & Osborn, 1969). Gels were stained with Coomassie Brilliant Blue.

Oxidation of the epidermal proteinase inhibitor

Methionine residues were oxidized to methionine sulphoxide residues (Johnson & Travis, 1979) by incubating the epidermal proteinase inhibitor (5 µg in 25 µl of water) with 5 µl of *N*-chlorosuccinimide (30 mg/ml in 0.1 M-Tris/HCl, pH 8.0) at 22°C for 30 min. The reaction was stopped by the addition of 20 µl of an aqueous methionine solution (2 mg/ml). Simultaneously, 5 µg of human α_1 -PI was oxidized by the same method for comparison.

Immunological reactivity

The reactivity of the epidermal proteinase inhibitor with rabbit antisera against rat or human serum α_1 -PI was analysed by the double-diffusion technique (Ouchterlony & Nilsson, 1973) on 1% agarose with phosphate-buffered saline (0.05 M- K_2HPO_4 /0.1 M-NaCl, pH 7.2).

Results

Purification of the epidermal proteinase inhibitor from newborn-rat epidermis

Inhibitory activity of the epidermal inhibitor against the cercarial enzyme, measured with [3H]-

elastin as a substrate, was detected in the fractions eluted from a Sephadex G-75 column at a position corresponding to approx. M_r 40 000–65 000 (Fig. 1). The inhibitor did not adsorb on CM-52 cellulose at pH 5.8, but the procedure helped to eliminate co-existing epidermal proteins in the same fractions (Table 1). The inhibitor adsorbed on the same column at pH 4.9 and was eluted out at 0.07 M-NaCl (Fig. 2). About 50-fold purification was achieved (Table 1). This material showed three protein bands on polyacrylamide-gel electrophoresis, under non-denaturing conditions, all of which showed inhibitory activity against purified porcine pancreatic elastase (Fig. 3b). Two major bands at M_r 40 000 and 53 000 were present on SDS/polyacrylamide-gel electrophoresis under reducing conditions (Fig. 3a). This

Fig. 1. Gel filtration by Sephadex G-75 chromatography of the epidermal proteinase inhibitor. Tris/HCl-soluble protein (212.4 mg) was applied to a column (2.5 cm $\times$ 90 cm), equilibrated, and eluted at 4°C with 0.02 M-Tris/HCl, pH 8.0, containing 0.14 M-NaCl. The flow rate was maintained at 15.2 ml/h, and 3.8 ml were collected in each fraction. The protein profile was analysed by A_{230} (—). The inhibitory activity of 100 µl of protein against 6 µg of *S. mansoni* cercarial enzyme (●) was measured with [3H]elastin as substrate, as described in the Experimental procedures section. The inhibitory fractions (46–66) were pooled for the next step.

Table 1. Summary of purification of a cercarial-proteinase inhibitor from newborn-rat epidermis. Inhibitory activity against the cercarial enzyme (6 µg) was measured by using [3H]elastin as substrate. The experimental conditions are described in the Experimental procedures section.

Purification step	Total protein (mg)	Protein (µg) required for 50% inhibition	Purification (fold)
Extract with 0.14 M-NaCl in 0.1 M-Tris/HCl, pH 8.0	212.4	396	1.0
Sephadex G-75	58.6	142	2.7
CM-cellulose			
pH 5.8	12.5	41	9.7
pH 4.9	2.4	8	49.5

fraction was used as the epidermal proteinase inhibitor for further enzymic assays and immunochemical characterization.

Characterization of the epidermal proteinase inhibitor

Specificity. Table 2 shows that the epidermal proteinase inhibitor was active against a wide range of serine proteinases. Clostridial collagenase (a

metalloproteinase) and papain (a cysteine proteinase) were not inhibited. Maximal activity was against leucocyte and porcine pancreatic elastase. Since porcine pancreatic elastase is readily available in a highly pure form, it was used for characterization of the inhibitor in some assays.

Effect of oxidation. The inhibitory activity of 5 µg of both the epidermal proteinase inhibitor and

Fig. 2. Cation-exchange chromatography of the epidermal proteinase inhibitor on CM-52 at pH 4.9

Freeze-dried protein (12.5 mg), obtained from previous CM-52 cellulose chromatography at pH 5.8 and exhibiting inhibitory activity, was applied to a column (1.5 cm × 12 cm) of CM-52 cellulose equilibrated with 0.05 M-sodium acetate, pH 4.9, at 4°C. After the column was washed with 3 bed-vol. of the buffer, adsorbed proteins were eluted with a linear 0–0.2 M-NaCl gradient (—) in 200 ml of buffer. The flow rate was maintained at 9.6 ml/h, and 3.2 ml were collected in each fraction. The absorbance profile was analysed at 230 nm (—). The inhibitory activity of 100 µl of the inhibitor against 6 µg of *S. mansoni* cercarial enzyme was measured with [³H]elastin as substrate (●). The bar (fractions 54–63) indicates where the epidermal proteinase inhibitor was collected.

Fig. 3. Polyacrylamide-gel electrophoresis of the epidermal proteinase inhibitor

(a) A 10 µg portion of reduced and denatured inhibitor was electrophoresed on an SDS/polyacrylamide gel as described in the Experimental procedures section. Arrows indicate position of: (1) phosphorylase b; (2) bovine serum albumin; (3) ovalbumin; (4) carbonic anhydrase. (b) The native inhibitor (30 µg) was electrophoresed on each of two non-SDS gels. One gel was stained with Coomassie Blue and scanned at 570 nm (—) in arbitrary units. The other gel was sliced into 2 mm-thick sections and extracted with 200 µl of 0.01 M-Tris/HCl, pH 8.0, at 4°C for 24 h. The inhibitory activity of 100 µl of this material against 5 µg of porcine pancreatic elastase (●) was assayed with [³H]elastin as substrate.

Table 2. Inhibitory activity of the epidermal proteinase inhibitor against various proteinases
Inhibition assays are described in the Experimental procedures section. Abbreviation used: NI, not inhibited at all by 20 µg of epidermal proteinase inhibitor.

Proteinase (µg)	Substrate (mg)	Epidermal inhibitor required for 50% inhibition (µg)
<i>S. mansoni</i> crude cercarial secretions (6–10)	Elastin (0.2)	10
<i>S. mansoni</i> partially purified cercarial proteinase (5)	Azocoll (5)	20
Porcine pancreatic elastase (5)	Elastin (0.2)	9
Human granulocyte elastase (3)	Elastin (0.2)	1
Bovine pancreatic trypsin (1)	Azocoll (5)	0.5
Bovine pancreatic α-chymotrypsin (3)	Elastin (0.2)	1
Clostridial collagenase (10)	Azocoll (5)	13
Papain (1)	Azocoll (5)	15
	Azocoll (5)	NI
	Bz-Arg-Nap·HCl (0.8)	NI

human α_1 -PI against 5 μ g of porcine pancreatic elastase (95 and 89% inhibition respectively) was completely lost after oxidation of methionine residues.

Stability. The epidermal proteinase inhibitor retained its activity after freeze-drying or storage at 4°C for 1 month, but lost 7% of its activity after 1 h at 40°C.

Immunological reactivity. Ouchterlony double-diffusion analysis showed no cross-reaction between 5 or 20 μ g of epidermal inhibitor and rabbit anti-serum to human serum α_1 -PI or rat serum α_1 -PI. Two different antisera preparations to rat serum α_1 -PI were tested. All three antisera showed precipitin bands when tested against human serum (20 μ g of α_1 -PI/well) or rat serum (22 μ g of α_1 -PI/well).

Discussion

Although epidermal cells constitute a formidable mechanical barrier, proteolytic secretions from the preacetabular glands of cercariae are capable of hydrolysing most macromolecular tissue barriers (see the introduction). The existence of an epidermal inhibitor of cercarial proteinase would therefore account in part for the inefficiency of *S. mansoni* infection. Our demonstration of a serine-proteinase inhibitor in epidermis is also noteworthy in the light of the recent demonstration of α_1 -PI in epithelial tissue (Bagdasarian *et al.*, 1981), suggesting a more widespread tissue distribution of such inhibitors than previously thought.

Many of the characteristics of the serine-proteinase inhibitor we have described resemble those of α_1 -PI. These include approximate M_r , class of proteinases inhibited and loss of inhibitory activity after oxidation with *N*-chlorosuccinimide (Roll & Glew, 1981; Johnson & Travis, 1979; Takahara *et al.*, 1980). However, the epidermal inhibitor did not cross-react with either anti-(human α_1 -PI) or two different preparations of anti-(rat α_1 -PI) antisera. This suggests that the epidermal inhibitor is either a unique proteinase inhibitor synthesized in the epidermis or α_1 -PI modified by a small ligand or amino acid change.

The epidermal cercarial proteinase inhibitor differs from previously described epidermal cysteine (thiol)-proteinase inhibitors (Hibino *et al.*, 1980; Jarvinen, 1978; Kominami *et al.*, 1981) in its spectrum of proteinase inhibition, M_r and behaviour on column chromatography. It also appears to be distinct from an epidermal trypsin inhibitor that does not inhibit chymotrypsin (Hibino *et al.*, 1981). The epidermal cercarial-proteinase inhibitor is similar in some properties to the trypsin-elastase inhibitor reported by Junnila *et al.* (1971) in human skin,

but further characterization of the latter inhibitor would be necessary to determine if they are identical.

Although our purpose in this investigation was to show a chemical barrier in skin to a specific parasite larva, the epidermal serine-proteinase inhibitor we have described may inhibit not only cercarial proteinase, but also proteinases from other infectious agents in the skin. It is furthermore possible that this epidermal inhibitory activity modulates proteinases released by inflammatory cells in skin diseases such as dermatitis herpetiformis and psoriasis.

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