THE MAJOR NEUTRAL PROTEINASE OF *ENTAMOEBA HISTOLYTICA*

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Although an infection with *Entamoeba histolytica* is most commonly asymptomatic, the potential for invasive, metastatic disease, coupled with its high prevalence, makes amoebiasis a major health problem throughout much of the world. Most amoebiasis-related mortality stems from extra-intestinal infection. In these cases, trophozoites penetrate the epithelial layer and lamina propria of the bowel mucosa, enter the bloodstream, and then disseminate to almost any organ or tissue, most commonly the liver.

The mechanisms that allow tissue penetration are not well understood. Ultrastructural and histopathological studies (reviewed in reference 1) have shown that, during invasion of the bowel wall, trophozoites are seen at the margin of ulcerative lesions adjacent to healthy tissue. Degeneration of epithelial cells adjacent to invading trophozoites and dissolution of the basement membrane of the mucosa have been observed (2–4). These findings suggest that histolytic and proteolytic factors may be elaborated to facilitate mucosal damage and invasion.

Two general types of histolytic factors have been proposed: cytotoxic factors which may directly damage cells, such as a secreted "amoebapore" ion channel (5, 6) and proteolytic enzymes, which may attack both cells and extracellular matrix.

The virulence[1] of axenically cultured *E. histolytica* strains has been correlated with the presence of proteolytic enzymes found on the amoeba surface, in secretions, or in extracts of whole trophozoites (9–14). Unfortunately, as none of these proteinases has been purified to homogeneity, important questions remain as to how many are present and how they may contribute to the pathogenesis of amoebiasis.

To determine what role secreted proteinases may play in tissue invasion, we assayed the degradation of extracellular matrix by live trophozoites and their secretory products, using an in vitro model of extracellular matrix successfully used to study other invasive parasites (15–18). These studies allowed us to

[1] For axenic amoebae in culture, "virulence" is defined in terms of ability to induce lesions in animals (7) or cytopathic effect on cell monolayers (8).

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determine which macromolecules were degraded in the type of interactive framework found in vivo, and to compare two strains of *E. histolytica* (HM-1 and HK-9) differing in virulence. We then undertook to purify and characterize the major proteinase secreted by trophozoites of the more virulent (HM-1) strain. We investigated the specific activity of the purified proteinase against type I collagen, elastin, fibronectin, and laminin, and studied its role in the cytopathic effect of virulent trophozoites on cells in culture. Finally, based on its ability to degrade a peptide substrate with arginine at the P-1 position, we assayed its possible function as a plasminogen activator.

Materials and Methods

Maintenance of Amoebae Cultures. Trophozoites of the axenic HK-9 and HM-1 strains were either obtained from the American Type Culture Collection (Rockville, MD) or were kindly provided by Dr. Frances Gillin, University of California, San Diego. Cultures were maintained in TYI-S-33 medium supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% bovine serum (Biofluids, Inc., Rockville, MD) for HK-9 or 15% bovine serum for HM-1, according to the methods of Diamond (19). For enzyme collections, amebae were plated at 5,000/ml in 25 cm^2 tissue culture flasks and harvested 3 d later. Amoebae were counted with a hemacytometer.

Preparation of Trophozoite Secretions and Extracts. Trophozoites harvested in log-phase growth (72 h after passage) were washed to remove serum and incubated in PBS with penicillin/streptomycin for 3 h at 37°C. Amoebae maintained >90% viability as judged by trypan blue exclusion and the absence of cytosolic alcohol dehydrogenase activity (20) in the PBS after incubation. Amoebae were centrifuged, and the resulting supernatant passed through a 0.45-μm filter. This was designated culture supernatant.

For extracts, trophozoites were resuspended in PBS with penicillin/streptomycin and lysed in a nitrogen cavitation bomb (Kontes Co., Vineland, NJ) after equilibration at 30 atm (3×10^6 N/m^2) for 1 h at 4°C. After centrifugation, the supernatant of this preparation was passed through a 0.45-μm filter and designated as soluble lysate.

The amount of protein was estimated by the method of Bradford (21).

Preparation of Radioactively Labeled Extracellular Matrix and Assay of Matrix Degradation. The method for preparation of extracellular matrix has been described in detail elsewhere (15). Briefly, R22 cells (derived from rat vascular smooth muscle) were grown in 24-well tissue culture plates in medium supplemented with [^{3}H]proline. Under these conditions, the cells produce a matrix of radioactively labeled glycoproteins, elastin, and collagen (primarily types I and III). After the cells were removed with NH_4OH, the insoluble matrix was washed and stored until used.

To assay for matrix degradation, live amoebae, lysate, culture supernatant, or purified proteinase fractions were incubated on the matrix at 37°C (reaction volume, 1 ml). Degradation was monitored by counting 50-μl aliquots of supernatant in a liquid scintillation spectrometer (LS-100; Beckman Instruments, Inc., Fullerton, CA) at several time points until a plateau was reached (24–48 h). The remaining reaction mixture was removed, and the specific degradation of noncollagenous glycoproteins, elastin, and collagen was estimated from the radioactivity released by sequential incubations of the residual matrix with trypsin, elastase, and collagenase, relative to controls (15, 22). Biochemical and ultrastructural analyses of matrices at each step of this sequential enzyme digestion have confirmed that trypsin degrades primarily noncollagenous glycoproteins (e.g., fibronectin), elastase degrades almost exclusively elastin, and collagenase degrades the remaining interstitial collagens (23, 24).

Purification of Proteinase Activity. To first determine how many proteolytic species were present in secretions, and their molecular weights under nonreduced conditions, 5 μl of crude supernatant and lysate were electrophoresed in 10% SDS-polyacrylamide gels copolymerized with 0.1% gelatin (Sigma Chemical Co., St. Louis, MO) (25). Gels were washed to remove SDS and incubated in buffer for 6–16 h at 37°C. After several cycles

of staining and destaining, proteolytic species were seen as clear bands on a Coomassie blue–stained background. This technique, as well as SDS-PAGE of boiled and reduced samples on silver-stained gels (26, 27), was used to monitor purification. Molecular weights were estimated from the migration of standards (Bio-Rad Laboratories; Richmond, CA).

Culture supernatant (5–10 ml at 0.5–5 mg/ml) was then dialyzed against buffer (20 mM Tris-HCl, 2 mM DTT, pH 7.5) and applied at a flow rate of 0.25 ml/min to a Mono Q anion-exchange column (Pharmacia Fine Chemicals, Piscataway, NJ). Protein was eluted with a linear salt gradient (20 mM Tris-HCl, 2 mM DTT, 200 mM NaCl, pH 7.5) at a flow rate of 0.5 ml/min using a GP-250 fast protein liquid chromatography (FPLC)[2] gradient programmer (Pharmacia Fine Chemicals). Fractions (1.5 ml) were collected and assayed for proteolytic activity against Azocoll, a general proteinase substrate (Sigma Chemical Co.), and against the synthetic peptide substrate Boc-arginine-arginine-4-amino-7-methylcoumarin (Z-Arg-Arg-AMC) (Enzyme System Products, Livermore, CA), which has been shown to be a substrate for the major neutral proteinase (12).

Fractions of peak activity from the anion-exchange column were pooled and dialyzed overnight against buffer (25 mM bis-Tris, 2 mM DTT, pH 7.1) and applied to a Mono P chromatofocusing column at a flow rate of 0.25 ml/min. About 3 ml at 0.5 mg/ml were typically injected. Protein was eluted at a flow rate of 0.5 ml/min over a pH gradient of 7–5 formed with 10% Polybuffer 74, pH 5.0 (Pharmacia Fine Chemicals). The gradient was generated with the GP-250 gradient programmer and continuously monitored with a pH meter. Fractions were collected and checked for activity as before.

2 mM DTT was used throughout as it enhanced activity two- to fourfold in assays and prolonged stability of the enzyme during storage. Even so, 5–25% of the activity was lost during each day in storage at −20°C. Storage at −70° or 4°C gave similar results.

Assays for Proteolytic Activity. All assays were performed in 100 mM Tris-HCl, 2 mM DTT, pH 7.4, unless otherwise noted.

Azocollytic activity was measured by incubation of 3 mg Azocoll with 10–100 μl of enzyme (1 ml reaction volume) for 3–16 h at 37°C. The tubes were then vortexed, microfuged, and the amount of degradation was determined from the absorbance of the supernatant at 540 nm (A_{540}). Control tubes were incubated without enzyme; bovine trypsin (5 μg; Sigma Chemical Co.) was used to determine the total available substrate.

The Z-Arg-Arg-AMC assay is based on the fluorescence of the cleaved AMC group at 460 nm when excited at 380 nm (28). Enzyme samples (5–25 μl) were added to a 4-μM solution of Z-Arg-Arg-AMC in buffer to a total volume of 2 ml. The rate of substrate hydrolysis at ambient temperature was determined from the rate of increase of fluorescence, monitored on a continuously recording spectrofluorometer (Aminco SPF-500; American Instrument Co., Silver Spring, MD). The scale was calibrated with a stock solution of 1.3 μM AMC (Sigma Chemical Co.).

Degradation of Fibronectin, Laminin, Elastin, and Type I Collagen. Aliquots (40 μg) of fibronectin (Bethesda Research Laboratories; Bethesda, MD), laminin (Bethesda Research Laboratories), or type I collagen (Vitrogen 100; Collagen Corp., Palo Alto, CA), diluted to 1 mg/ml in buffer with 150 mM NaCl, were mixed with 40 μl of Mono P–purified enzyme or 10–20 μl of culture supernatant. Volumes were brought to 80 μl with 100 mM Tris, pH 7.2, and DTT was added to a final concentration of 2 mM. Samples were incubated for 3.5 h (fibronectin and collagen) or 20 h (laminin) at 37°C, along with matched amounts of substrate or enzyme alone as controls. Collagen was also incubated with 0.5 μg of TPCK-trypsin (Worthington Biochemical Corp., Freehold, NJ) to assay for nonspecific degradation.

Reactions were terminated with 2× sample buffer and analyzed by SDS-PAGE (laminin with 5% and 10% gels; fibronectin with 7% gels; collagen with 10% gels) for evidence of degradation; 80 μl was loaded into each lane.

Elastase activity was assayed as previously described (25). Briefly, 200 μg of $NaB[^3H]_4$-labeled elastin (Elastin Corp., St. Louis, MO) was incubated with 100 μl of culture

[2] *Abbreviations used in this paper:* BHK, baby hamster kidney; FPLC, fast-protein liquid chromatography; NEM, *N*-ethyl-maleimide; TLCK, tosyl-lysyl-chloromethyl ketone; Z-Arg-Arg-AMC, Boc-arginine-arginine-4-amino-7-methylcoumarin.

TABLE I
Inhibition of Mono P–purified Enzyme

Inhibitor	Final concentration	Activity as percent of control* (without inhibitor)	
		Culture supernatant*	Mono P–purified enzyme‡
N-ethylmaleimide (NEM)	5 mM	5	0
Iodoacetate	3 mM	0	0
Leupeptin	10 μM	68	16
	50 μM	ND	0
α_1-Proteinase inhibitor	100 μg/ml	58	0
TLCK	0.1 mM	0	0
Aprotinin (trasylol)	100 μg/ml	100	100
EDTA	10 mM	100	100
PMSF	2 mM	100	100
1,10-Phenanthroline	2 mM	100	100
Pepstatin A	100 μg/ml	100	100
Bovine serum	0.5% (vol/vol)	0	0
Soybean trypsin inhibitor (SBTI)	100 μg/ml	100	100

ND, not determined.
* By Z-Arg-Arg-AMC assay, except PMSF and serum, which were by Azocoll assay.
‡ By Azocoll assay.

supernatant or 100 μl of Mono P–purified enzyme in buffer (reaction volume, 300 μl) for 19 h at 37°C. Total available substrate was determined by digest with pancreatic elastase (Sigma Chemical Co.).

Plasminogen Activator Assay. Plasminogen activator activity was determined by the method of Unkeless et al. (29) as modified by Aggeler et al. (30). Briefly, 24-well plates were coated with ^{125}I-labeled fibrinogen and incubated with calf serum for 2 h at 37°C to convert fibrinogen to fibrin. Plasminogen (280 ng; final concentration, 6 nM) was added in 50 mM Tris-HCl (pH 7.8) with 100 μl of culture supernatant or amoebic lysate, or 50 μl of Mono P–purified enzyme, to a final volume of 0.5 ml. Controls were incubated without plasminogen added. Aliquots of supernatant (50 μl) from quadruplicate wells were counted in a gamma counter (Auto-Gamma 500; Packard Instrument Co., Inc., Downers Grove, IL). Total available fibrin was determined by trypsin digest. Urokinase (10 Ploug units; Calbiochem-Behring; La Jolla, CA) was assayed for comparison. Results were expressed as the percentage of total available fibrin degraded, less the percentage degraded by the corresponding plasminogen-free control.

Effects of Inhibitors. Inhibitors were preincubated with the enzyme at the indicated concentrations (Table I) for 20 min at ambient temperature; substrate was added and proteolytic activity was assayed against Z-Arg-Arg-AMC. All inhibitors were obtained from Sigma Chemical Co. except pepstatin A (Transformation Research, Inc., Framingham, MA) and bovine serum (Biofluids Inc.). Tosyl-lysyl-chloromethyl ketone (TLCK) was prepared as a stock solution in DMSO. PMSF, pepstatin A, and 1,10-phenanthroline stock solutions were made up in ethanol. Other inhibitors were made up in water or assay buffer. An equal volume of DMSO or ethanol alone was tested with the enzyme, and inhibition relative to the appropriate solvent control was calculated.

pH profile. Crude or chromatography-purified enzyme was assayed for proteolytic activity against Z-Arg-Arg-AMC as described. Acetate buffer (100 mM) was used for pH 4.0–6.0, 100 mM phosphate buffer for pH 6.8–8.0, and 100 mM Tris for pH 6.0–10.0. All buffers included 2 mM DTT.

Assay Effects on Cultured Cells. Confluent monolayers of baby hamster kidney (BHK)

cells (obtained from the UCSF Cell Culture Facility), grown in 24-well tissue culture plates in DME supplemented with 2 mM glutamine, penicillin/streptomycin, and 6% FCS, were washed to remove serum and incubated at 37°C with 200 μl of culture supernatant or 400 μl of Mono P–purified enzyme. (Medium was added to a total volume of 1 ml/well.) Duplicate wells were compared with control wells of DME-H21 diluted with matched amounts of PBS or buffer, with and without serum. Cells were examined with an inverted microscope for evidence of cytopathic effects (cell rounding, cell detachment, and cell lysis) over the next 3 h.

Results

Extracellular Matrix Degradation. Live HM-1 trophozoites (2×10^5) degraded 11% of the extracellular matrix in 26 h, including 50% of the glycoproteins and 10% of the collagens, but none of the elastin. The same number of HK-9 trophozoites degraded only half as much of the matrix.

Culture supernatant (1 ml; 1.3 mg protein) degraded 20% of the matrix in 22 h, including >80% of the available glycoproteins but little of the elastin or collagen. Lysate (1 ml; 1.5 mg protein) gave similar results. Mono P–purified enzyme (340 μl; 0.2 mg protein) degraded 24% of the matrix in 26 h, primarily glycoproteins (>90%).

Activity of Unpurified Enzyme. Both lysate and culture supernatant had activity against Azocoll and the Z-Arg-Arg-AMC peptide substrate. Lysate from HM-1 trophozoites had twice as much proteolytic activity as that from the same number of HK-9 trophozoites on the Azocoll substrate. Culture supernatant from HM-1 trophozoites was five times more active than that from an equal number of HK-9 trophozoites. Therefore, the more active HM-1 strain was used for the subsequent purification and characterization of neutral proteinase activity.

SDS-PAGE and SDS-gelatin-PAGE showed that although both lysate and culture supernatant were complex mixtures, the major proteolytic activity was found at M_r 50,000–70,000 (Fig. 1). Tested over a pH range from 4 to 10, both lysate and culture supernatant had a peak of proteolytic activity at pH 7.5 (Fig. 2). This activity was completely inhibited by *N*-ethyl-maleimide (NEM), iodoacetate, and TLCK, but not affected by PMSF, EDTA, pepstatin, or 1,10-phenanthroline at the concentrations tested (Table I).

Culture supernatant (100 μl) activated plasminogen sufficient to degrade 19%

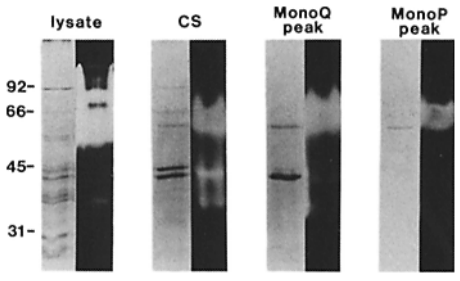

FIGURE 1. SDS-PAGE and SDS-gelatin-PAGE analysis of amoeba protein and proteinase composition. Silver-stained gels of reduced enzyme samples (*left*) are paired with unreduced, Coomassie-stained gelatin substrate gels (*right*) for each fraction. CS, culture supernatant. Molecular weight markers ($M_r \times 10^{-3}$) are indicated.

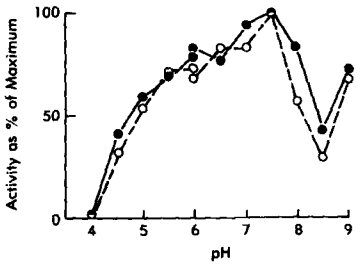

FIGURE 2. pH profile of culture supernatant and lysate. 10 μl of culture supernatant (CS) or lysate was assayed against Z-Arg-Arg-AMC in 100-mM acetate (pH 4–6) or 100-mM Tris (pH 6–9) buffers as described in Materials and Methods. Results are expressed as the percentage of maximum proteolytic activity observed. ●, lysate; O, CS.

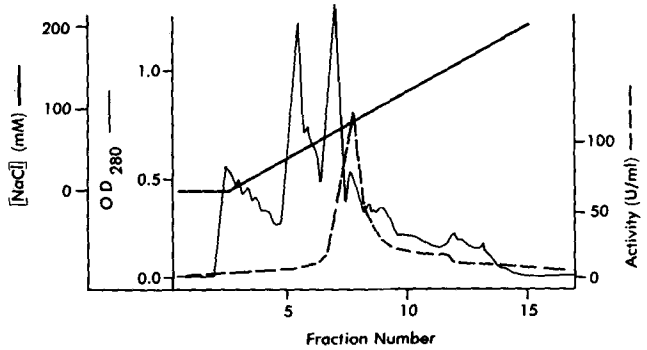

FIGURE 3. Anion-exchange (Mono Q) chromatography of culture supernatant. Protein was eluted with a linear salt gradient as described in Materials and Methods. Proteolytic activity against Z-Arg-Arg-AMC is shown. The activity profile by Azocoll assay was identical. U, units of activity by AMC assay (μmoles substrate cleaved × $liter^{-1}$ × min^{-1}). OD_{280} was measured in a 3-mm path length flow cell.

of the available fibrin in 3 h. The same amount of lysate degraded 25% of available fibrin, and 10 Ploug Units of urokinase degraded 38%.

Our results with unpurified material suggested that the major proteinase of *E. histolytica* was a thiol enzyme that was secreted by trophozoites and that would be active in the neutral pH environment of the host. Our subsequent work was aimed at purifying and further characterizing this activity. Insofar as we observed no significant qualitative difference between the proteolytic activities of lysate and culture supernatant, and because there were considerably fewer major proteins in culture supernatant (Fig. 1), we chose the latter as the starting material for further purification.

Chromatographic Purification and Activity of Purified Fractions. Activity eluted from the Mono Q anion-exchange column as a single peak at a salt concentration of 60–80 mM (Fig. 3). When peak fractions were pooled and applied to the Mono P chromatofocusing column, activity eluted in a single peak at pH 5.9–6.2 (Fig. 4). SDS-PAGE indicated a subunit molecular weight of ~56,000 (Fig. 1).

Overall, the specific activity of Mono P–purified enzyme was increased 10-fold over that of culture supernatant, with 19% of total activity recovered (Table II).

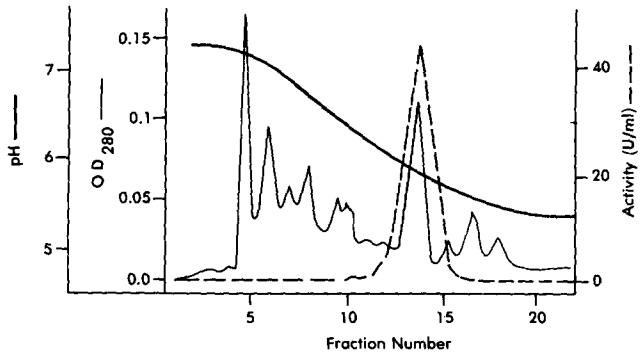

FIGURE 4. Chromatofocusing (Mono P) chromatography of peak fractions pooled from Mono Q-purified enzyme. Protein was eluted with a pH gradient as described in Materials and Methods. Proteolytic activity of fractions against Z-Arg-Arg-AMC is shown. OD_{280} was measured in a 3-mm path length flow cell.

TABLE II

Purification Table of the Neutral Thiol Proteinase

Step	Total protein	Specific activity	Enrichment	Total activity	Recovery
	mg	*U/mg*	*-fold*	*U*	%
Culture supernatant	4.7	57	1	268	100
Anion exchange (Mono Q)	1.2*	130*	2*	220‡	82‡
Chromatofocusing (Mono P)	0.022*	590*	10*	50‡	19‡

Values are for a representative purification, assayed for activity against Z-Arg-Arg-AMC. U, units of activity (μmoles AMC cleaved/liter/min.

* For peak fractions.

‡ Total for all fractions recovered.

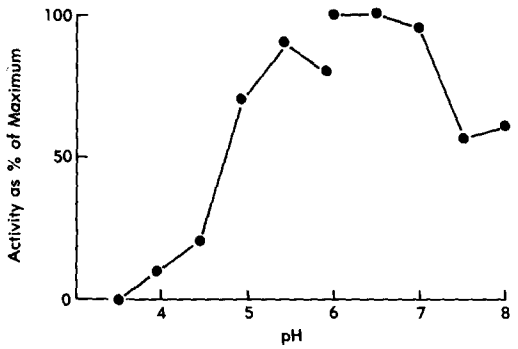

FIGURE 5. pH profile of Mono P-purified enzyme. 10 μl of enzyme was assayed against Z-Arg-Arg-AMC in 100-mM acetate (pH 3.5–6) or 100-mM phosphate (pH 6–8) buffers with 2 mM DTT. Results are expressed as the percentage of maximum proteolytic activity observed.

When assayed in phosphate buffer, Mono P-purified enzyme had maximal activity against Z-Arg-Arg-AMC at pH 6–7 (Fig. 5). In Tris buffer, there was a broader peak from pH 7–9.5 (data not shown). The proteolytic activity of

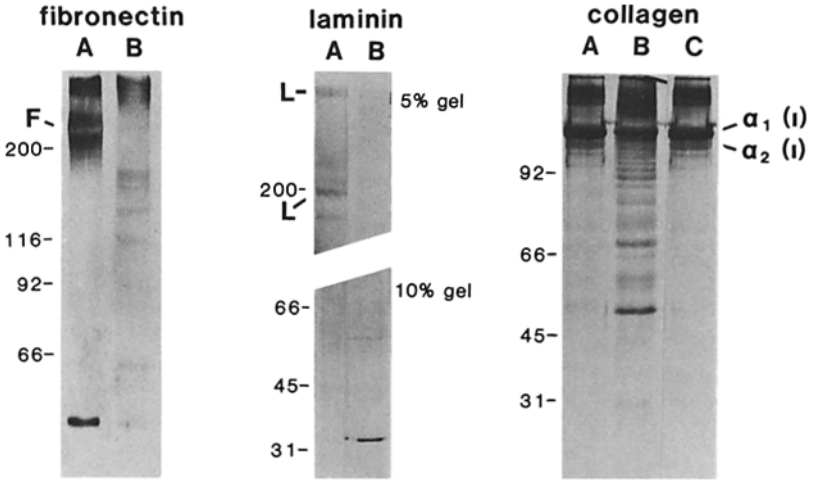

FIGURE 6. SDS-PAGE analysis of connective tissue macromolecular degradation by Mono P–purified enzyme. Target species in these reduced gels are indicated: fibronectin (*F*), laminin (*L*), and the type I collagen subunits [α_1 (I) and α_2 (I)]. Laminin was run on both 5% gels (to show laminin degradation) and 10% gels (to show cleavage products). (*A*) Substrate alone; (*B*) substrate and enzyme. Collagen was also incubated with trypsin (*C*), showing lack of nonspecific proteolysis. Control lanes of enzyme alone had no visible staining. Molecular weight markers ($M_r \times 10^{-3}$) are indicated.

purified enzyme was completely inhibited by NEM, iodoacetate, leupeptin (50 μM), α_1-proteinase inhibitor, TLCK, and serum at the concentrations tested (Table I).

Analysis by SDS-PAGE indicated that the purified neutral thiol proteinase was able to degrade purified laminin, fibronectin, and type I collagen (Fig. 6). Collagen was cleaved into many fragments; the characteristic cleavage pattern of bacterial collagenase, with major fragments of M_r 75,000, 50,000, and 25,000, was not apparent. Culture supernatant also degraded these macromolecules (not shown).

Mono P–purified enzyme (50 μl, ~1 μg) activated plasminogen sufficient to degrade 3.4% of the available fibrin in 2.5 h.

There was no degradation of [^{3}H]elastin by culture supernatant or purified enzyme.

Effects on Cultured Cells. Both culture supernatant and purified enzyme caused a marked effect on cell adhesion, which was evident within 45 min. At 2 h, >95% of the cells had rounded up and detached (Fig. 7). In control wells, <25% of the cells had detached. However, no lysis or osmotic damage to cells was observed. Detached cells, when washed and replated, reattached and exhibited normal morphology.

Discussion

We have purified to homogeneity a neutral proteinase that is secreted by *E. histolytica* trophozoites and can also be identified in the soluble fraction of trophozoite lysate (12). Because of its inhibition profile and potentiation by DTT, we consider the enzyme to be a thiol proteinase. Like vertebrate cathepsin B (31), it is active against a synthetic peptide substrate with arginine at P-1 and P-

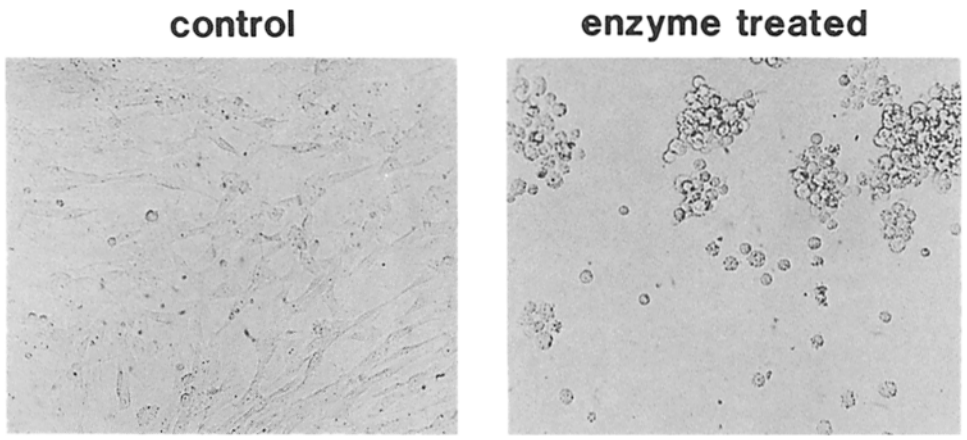

FIGURE 7. Effect of purified enzyme on BHK cells in culture. Cells rounded up and detached in wells treated with Mono P–purified enzyme (*right*). The same results were seen with culture supernatant. All control wells were similar to each other. Photographs were taken 90 min after enzyme was added.

2. The enzyme has a subunit molecular weight of ~56,000 (by SDS-PAGE) and an approximate pI of 6 (by chromatofocusing).

The enzyme we have purified probably accounts for the thiol proteinase activity of crude extracts and secretions reported previously (9, 10, 32, 33). Although estimates of molecular weight differ, it probably is the same neutral proteinase as that partially purified from a different strain of *E. histolytica* by McLaughlin and Faubert (33), and the same as the cathepsin B-like proteinase partially purified from HM-1 lysate by Lushbaugh et al. (14). This latter enzyme shares three important characteristics with the proteinase we have purified: (*a*) ability to degrade peptides with arginine at P-1 and P-2, (*b*) inhibition by thiol proteinase inhibitors and potentiation by DTT, and (*c*) "cytopathic" effect on cells in culture. The observed differences between the two enzymes in pH optima are not great and could be explained by the use of different buffers or assay conditions. The reported molecular weights, however, differ considerably (16,000 vs. 56,000). In addition to the major band at M_r 16,000, Lushbaugh et al. (14) reported four other protein species between M_r 35,000 and 48,000 in the same preparation by SDS-PAGE. Our (unreduced) substrate gels of lysate show significant proteolytic activity (at neutral pH) only between ~M_r 50,000 and 70,000 (Fig. 1), and silver-stained gels of active fractions partially purified from lysate showed only two major bands at 47,000 and 60,000 (unpublished data). It is possible, therefore, that one of the higher molecular weight species seen by Lushbaugh et al. (14) may be the active one, rather than the M_r 16,000 band. Another possibility is that during the longer purification protocol required for lysate, proteolysis generated lower molecular weight products, which can sometimes retain activity (see, for example, reference 25). Proteolysis may have been minimized with our more rapid FPLC purification protocol.

The neutral thiol proteinase we have purified is clearly distinct from the membrane-associated metallocollagenase reported by Munoz et al. (34). That

enzyme was not secreted, and was inhibited by EDTA but not by NEM. The enzyme we have purified is also different from the acid proteinase partially purified from lysate by Scholze and Werries (35), which had a pH optimum of 4, pI of 4.9, and apparent M_r of 21,000 (estimated by size-exclusion chromatography). McLaughlin and Faubert (33) also described an acid proteinase (peak activity at pH 3.5) with an estimated M_r of 41,000 (also by size exclusion chromatography), but its activity against hemoglobin as substrate was unaffected by DTT, iodoacetamide, or *p*-chloromercuribenzoate. Feingold et al. (36) reported the partial purification of a trophozoite enterotoxin, which had a cytopathic effect on BHK cells, and degraded casein but not Azocoll. It had an M_r of 30,000–50,000, and its effect on cells was blocked by *p*-chloromercuribenzoate, iodoacetamide, and serum.

That the neutral thiol proteinase is important for the pathogenesis of extraintestinal amoebiasis was first suggested by our observations that it is secreted by trophozoites and active at neutral pH. Furthermore, HM-1 strain trophozoites secrete more of this proteinase than the less virulent HK-9 strain. Other investigators have also correlated strain virulence with proteolytic activity (9–14, 32, 37).

The results of assays of crude and purified enzyme against an in vitro model of extracellular matrix, as well as against purified type I collagen, laminin, and fibronectin, suggest how this enzyme may play a key role in both invasion and tissue damage by trophozoites. Gadasi and Kobiler (10) showed that crude lysate from HM-1 trophozoites degraded fibronectin. Our results with purified enzyme confirm this finding and identify the proteinase responsible for this activity. Furthermore, we have shown that this enzyme cleaves laminin, which plays a critical role in anchoring epithelial cells to underlying basal lamina, as well as in maintaining the structural integrity of the basement membrane.

It has been proposed that proteinases are involved in the observed cytopathic effects of virulent trophozoites on cells in culture (10, 13, 14, 38). Our demonstration of cell detachment and rounding up after exposure to the purified enzyme probably reflects the ability of the enzyme to degrade anchoring proteins, such as fibronectin and laminin. Some investigators have used the term "cytopathic effect" to describe this cell detachment (10, 36, 42). This phenomenon, however, must be distinguished from cell lysis or osmotic damage (39), which we did not observe. Lysis per se may require the activity of other cytotoxic factors, such as the amoebapore (5, 6). Nevertheless, proteinase-mediated detachment of epithelial cells from the bowel mucosa may be an important pathogenetic mechanism in amoebiasis.

The activity of the thiol proteinase against type I collagen corresponds to that reported for crude secretions and lysate by Gadasi and Kessler (9). While the metallocollagenase reported by Munoz et al. (34) produced specific cleavage fragments of M_r 75,000, 50,000, and 25,000, the neutral thiol proteinase cleaves collagen into multiple fragments, as does cathepsin B. Mammalian cathepsin B further degrades the collagen fragments produced by vertebrate collagenase (40). Similarly, the neutral thiol proteinase of *E. histolytica* could act in concert with the membrane-associated metallocollagenase to degrade interstitial collagen. This could explain earlier observations that, after initial collagen fragmentation

by intact trophozoites, further breakdown was inhibited by NEM and stimulated by DTT (34).

E. histolytica trophozoites have thiol groups on or accessible to their external surface, which appear to be necessary for cell survival (41). Thus, the trophozoite may provide in its pericellular space the optimal reducing environment for the activity of the enzyme we have characterized. This could be one reason why the cytopathic effect of *E. histolytica* is optimal when apparent contact between the organism and epithelial cells takes place (39). Even without contact, the closer the organism approaches the substrate, the better the environment may be for the activity of its secreted enzyme. Close apposition of trophozoites to cells would also reduce the accessibility of inhibitory serum proteins to the site of enzyme-substrate interactions (42).

In addition to degrading host extracellular matrix and basement membrane macromolecules, the enzyme we have purified is a plasminogen activator. We tested for this activity because the enzyme cleaved synthetic peptides with arginine at P-1, as do other plasminogen activators. Therefore, aside from directly mediating tissue damage, the amoebic enzyme may potentiate host proteinases by activating plasmin. Plasmin can then amplify its own activation, as well as activate latent tissue collagenases.

Although our characterization of the neutral thiol proteinase of *E. histolytica* suggests its importance as a virulence factor, it remains clear that the pathogenesis of amoebiasis is multifactorial. In the neutral pH environment of the host, at least two amoebic proteinases are active: the enzyme we have purified and the membrane-associated metallocollagenase. Other cytotoxic factors, such as the amoebapore, may be necessary for cell lysis. Host factors, including associated gut flora (36, 43), diet and nutritional status, and immunologic competence and history (44), are no doubt also involved.

Proteolytic enzymes may be important in ways other than as mediators of tissue invasion and destruction. For example, they might degrade immunoglobulins, generate (or destroy) peptides chemotactic for host inflammatory cells, or activate the kallikrein/kinin pathway to alter vascular permeability. We are currently investigating whether the neutral thiol proteinase potentiates these or other reactions, because it shares with kallikrein, plasmin, and complement proteinases the ability to cleave peptides containing arginine.

Summary

FPLC anion-exchange and chromatofocusing chromatography were used to purify the major neutral proteinase from secretions of axenically cultured *Entamoeba histolytica* trophozoites. HM-1 strain trophozoites, which were more proteolytically active than the less virulent HK-9 strain, were used for purification of the enzyme. It is a thiol proteinase with a subunit M_r of ~56,000, a neutral pH optimum, and a pI of 6. The importance of this enzyme in extraintestinal amoebiasis is suggested by its ability to degrade a model of connective tissue extracellular matrix as well as purified fibronectin, laminin, and type I collagen. The enzyme caused a loss of adhesion of mammalian cells in culture, probably because of its ability to degrade anchoring proteins. Experiments with a peptide substrate and inhibitors indicated that the proteinase preferentially binds pep-

tides with arginine at P-1. It is also a plasminogen activator, and could thus potentiate host proteinase systems.

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