Title

Identification, characterization and purification of a secreted cysteine protease from naegleria species and its role in the pathogenesis of primary amebic meningoencephalitis

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Identification, Characterization and Purification of a Secreted Cysteine Protease from *Naegleria* Species and Its Role in the Pathogenesis of Primary Amebic Meningoencephalitis

by

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A Thesis

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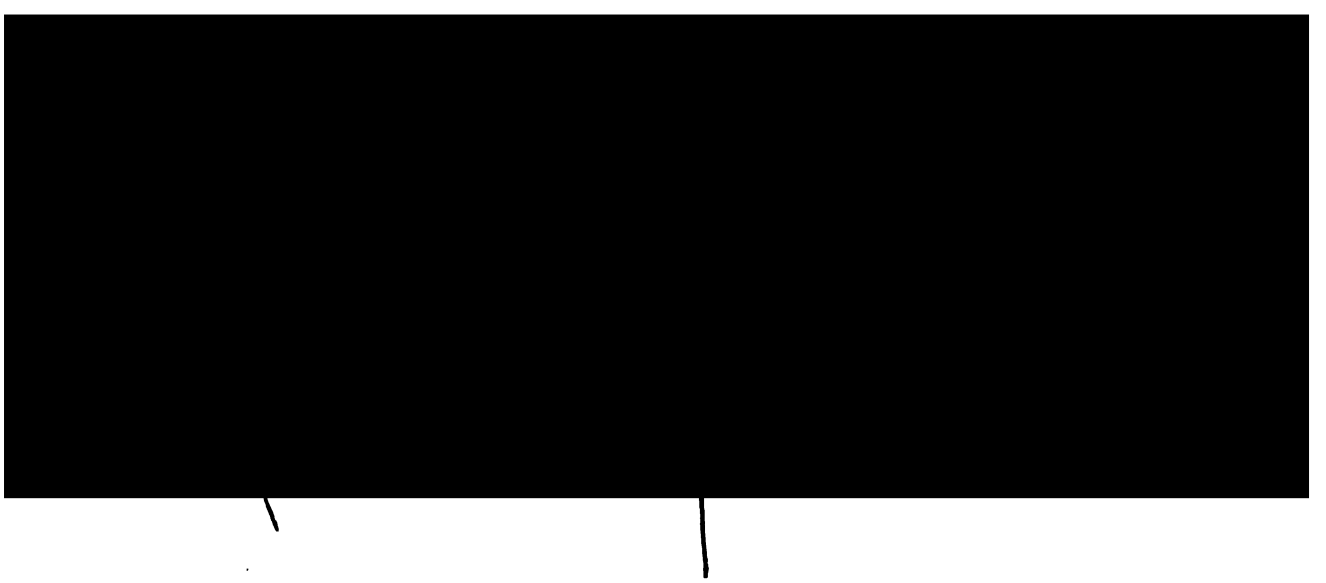

Abstract

Naegleria fowleri, a free-living ameba of fresh water ponds, is the etiologic agent of primary amebic meningoencephalitis (PAM). Of the several hundred reported cases all but two have resulted in fatalities, and little is known of the factors important for the pathogenesis or treatment of PAM. *N. fowleri* rapidly invades the central nervous system via the nasal mucosa and cribiform plate and we hypothesized that, by analogy with other pathogenic protozoa, it secretes a protease which aids in invasion of these tissues. *N. gruberi* is a non-pathogenic species which, unlike its pathogenic relative, grows at 30°C but not 37°C. Assays of media and phosphate-buffered saline conditioned by replicating amebae revealed secreted protease activity from both species. Purification and characterization of the proteases from conditioned media of both species revealed approximately 30 kD proteins with substrate and inhibitory profiles consistent with that of the cysteine class of proteases. N-terminal sequence analysis of the purified protein from *N. gruberi* was obtained. The sequence was homologous to sequence motifs of known cysteine proteases and provided a basis for the design of oligonucleotide primers which we are currently using in the polymerase chain reaction in order to isolate the gene. To determine the function of this protease in disease, we first confirmed that whole amebae of both species degraded extracellular matrix synthesized by mammalian (R22) cells and, as had been reported by previous investigators, was cytopathic to cultured

mammalian (BHK) cells. We then showed that the cysteine protease isolated from amebae also degraded the R22 matrix, and caused the cytopathic effect. Z-Phe-Ala-flouromethyl ketone (ZFA-fmk), a transition state analogue, was found to inhibit the secreted protease activity in nanomolar concentrations. ZFA-fmk inhibited the ability of whole amebae to degrade the R22 extracellular matrix and protected BHK cells from the cytopathic effect caused by either whole amebae or the purified enzymes. In sum, our results indicate that *N. fowleri* secretes a 27-30 kD cysteine protease which may be important for its ability to rapidly invade nasal mocosa and neural tissue. *N. gruberi* is non-pathogenic, we hypothesize, not because it lacks a similar protease, but because it is unable to grow at ambient body temperature. ZFA-fmk, a protease inhibitor already shown to have negligible toxicity to animals, may now be tested as a potential chemotherapeutic agent for PAM.

Introduction

The genus *Naegleria* consists of free-living amebae widely distributed throughout the world. One species of this genus, *N. fowleri*, is the causative agent of primary amebic meningoencephalitis (PAM) (for reviews, see ref 1,2,3). This species, which habits warm fresh water ponds, exhibits a unique mode of entry into the central nervous system, via intranasal inoculation in the unsuspecting swimmer. Invasion through the nasal mucosa, cribiform plate and finally the base of the brain, then occurs. While occasional surviviors have been reported (4), treatment is generally inadequate, the patient typically dying of complications of fulminant inflammation and hemorrhage of the brain and meninges. The virulence factors important for the pathogenesis of PAM are a matter of debate (1) and several have been hypothesized, including a membrane-associated pore-forming protein (5), phospholipases (6), undefined hemolytic factors (7,8,9), or direct cell-cell contact cytopathogenicity (1). The necessary components for tissue invasion, however, are not precisely defined, and remain potential targets for chemotherapy.

One might expect that in order for invasion through tissue layers to occur, histolytic enzymes must be produced by the pathogen. In this regard, we hypothesized that proteolysis may be one component of tissue invasion. Thus we attempted to identify proteases, particularly secreted ones, that were present in *Naegleria* species.

Proteases, enzymes which catalyze the hydrolysis of peptide bonds, are divided into peptidases, enzymes which degrade from the end of the peptide chain, and endopeptidases (proteinases), which catalyze cleavage of an internal peptide bond. Endopeptidases, in turn, are separated into four major classes according to the important chemical groups present at the active site: cysteine (thiol), serine, metallo, and aspartyl (carboxy). Proteases are ubiquitous in nature, participating in processes such as prohormone processing, blood coagulation and immune reactions (10). Additionally, proteases have recently been found to play a number of critical roles in the pathogenesis of parasitic diseases, in particular facilitating the invasion of host tissues (10). As an example, the cercariae of *Schistosoma mansoni* are thought to undergo their intial stages of human infection by means of an elastinolytic serine protease which enables penetration and degradation of skin proteins (11).

Of proteases characterized from parasitic protozoa, cysteine proteases represent the most abundant class. To date cysteine proteases have been identified in the parasitic protozoan genera *Leishmania*, *Trypanosoma*, *Trichomonas*, *Giardia*, *Plasmodium* and *Entameoba* (reviewed in ref. 12). Their presence across so many genera may imply a central role in protozoan metabolism and/or pathogenicity. A relevant example is the secreted cysteine protease of *Entamoeba histolytica*, the causative agent of amebiasis in humans. Purified from secretions of growing amebae, it was found to mimic the cytopathic effect (the destruction of target mammalian cells) of whole amebae *in vitro* (13). Furthermore, a

correlation between the amount of protease secreted and the degree of cytopathic effect produced by different strains was found (14). 80% of patients with histories of invasive amebiasis produced antibodies to the cysteine protease, and the presence of protease activity in clinical isolates of *E. histolytica* strains was highly correlated with the presence of invasive disease (15). The recent introduction of potent and specific cysteine protease inhibitors has raised hopes they might be useful as antiparasitic chemotherapeutic agents. The floromethyl ketone inhibitor ZFA-fmk was found to inhibit the production of a cytopathic effect by pathogenic *E. histolytica* against mammalian cells (14).

Cysteine proteases have also been implicated in other examples of tissue destruction in disease. Several types of malignant tumor cells which demonstrate the ability to invade into and through normal tissue (as occurs during metastasis) have been shown to release cysteine proteases, particularly of the cathepsin L and B classes (16,17,18). Additionally, cysteine protease activity has been reported to be present *in situ* in both experimental (19) and human (20) arthritis, using specific synthetic substrates histochemically. This activity appears to be important for tissue destruction, since selective inhibition of cysteine protease activity by ZFA-fmk suppressed the degree of inflammation and decreased damage to cartilage in an experimental rat model of arthritis (19).

In this report, we describe the identification, purification and characterization of a secreted cysteine protease of *Naegleria*

species. On the basis of *in vitro* studies of extracellular matrix digestion and direct cytopathogenicity, we hypothesize that it is important for tissue invasion by the amebae, and thus the pathogenesis of PAM. Proteases from the pathogenic species, *N. fowleri*, and from the non-pathogenic species, *N. gruberi* (which is unable to grow at temperatures greater than 30°), are described and we further hypothesize that the inability of the non-pathogenic species to grow at ambient body temperature, not a lack of tissue-destructive proteases, prevents it from being pathogenic.

Materials and Methods

Growth of Naegleria *cultures.* Trophozoites of axenic *N. fowleri* and *N. gruberi* species were maintained at 37° C and 30° C, respectively, in H-4 medium consisting of Page ameba saline, glucose, proteose peptone, yeast extract and liver extract. Hemin (1 μg/ml) was added in place of fetal calf serum as described previously (21). Amebae were passaged every 3-5 days to maintain log phase growth. Penicillin G (100 units/ml) and streptomycin (100 μg/ml) were added to prevent bacterial contamination. Amebae were grown in Corning 150 cm^2 tissue culture flasks (Corning Co., Corning NY). Amebae were counted by shaking the flasks vigorously to dislodge them from the bottom of the flask, and a sample of this preparation was then added to a hemacytometer.

Preparation of trophozoite secretions. Secretions were prepared based on the method of Keene *et. al.* (13). Briefly, media was poured off of amebae grown to confluence at log phase (72 hours after passage). Phosphate-buffered saline (PBS) was then added, and left for 24 hours at the appropriate temperature. The cells remained viable after the PBS incubation as judged by counting before and after the incubation with a hemacytometer. The conditioned PBS was then gently poured into a centrifuge tube and remaining trophozoites were sedimented at 2000*g* for 10 min. The supernatant was assayed for protease activity and used for subsequent experiments as described below. The amount of protein was estimated by the method of Bradford (22).

Protease assays. Aminomethylcoumarin (AMC) peptide substrates (Enzyme Systems Products, Livermore, CA) were dissolved in DMSO as a stock solution (10mM) and added to give a final concentration of 5 μM in the reaction buffer. The peptide-AMC assay is based on the fluorescence of the cleaved AMC group at 460 nm when excited at 380 nm (23). Enzyme samples (5-10 μl) were added to 1.5 ml of reaction buffer, containing 100 mM Tris-Cl, pH 7.5, 1mM EDTA, 2mM DTT, and 5 μM benzyloxycarbonyl-valine-leucine-arginine-AMC (Z-VLR-AMC) in a glass cuvette, and initial reaction velocity was immediately measured in a continuously recording spectrofluorimeter (Aminco SPF-500; American instrument Co. , Silver Spring MD) as previously described (13). The scale was calibrated with a standard solution of 1.3 μM AMC (Sigma Chemical Co., St. Louis, MO) dissolved in DMSO.

SDS polyacrylamide gel electrophoresis. To first determine the size of the proteolytic species present in *Naegleria,* gelatin gel electrophoresis was performed as described previously (11). Briefly, 20 μl of crude PBS secretions were loaded onto 10% SDS-polyacrylamide gels copolymerized with 0.1% gelatin (Sigma Chemical Co.). Gels were washed three times in distilled water to remove SDS and incubated in buffer (100mM Tris-Cl pH 7.5, 1mM EDTA, 2mM DTT) overnight at 37° or 30° C. After staining with Coomassie blue and subsequent destaining, proteolytic species were seen as clear bands on a blue background. Standard SDS-PAGE of heated and reduced samples (boiled for 10 min in 20mM DTT) was

also performed on 10-12% acrylamide gels (20 milliamps for 1 hour) as described previously (24). Molecular weights were estimated from the migration of protein standards (14.4-97.4 kD, Bio-Rad Laboratories, Richmond, CA).

Purification of protease activity. Culture supernatant (50 mls of secretions from 10^8 ameobae) was then dialyzed overnight against 4L buffer (20 mM Tris-Cl, pH 8, 4°C) and applied at a flow rate of 1 ml/min to a Mono-Q anion-exchange column (Pharmacia Fine Chemicals, Piscataway, NJ). Protein was eluted with a linear salt gradient of 0-500mM NaCl using 20 mM Tris-Cl, pH 8.3, 500mM NaCl as an eluting buffer at a flow rate of 1 ml/min using a GP-250 fast protein liquid chromatography (FPLC) gradient programmer (Pharmacia Fine Chemicals). The flow-through and fractions collected (1 ml) were assayed for protease activity using the AMC assay. Peak fractions were pooled and dialyzed against buffer (25 mM bis-Tris, pH 6.3) and applied to a Mono-P chromatofocusing column at a flow rate of 1 ml/min. Protein was eluted at the same flow rate over a pH gradient of 6-4 formed with 10% Polybuffer 74 (Pharmacia Fine Chemicals) in 25 mM bis-Tris, pH 4.0. Flow-through and fractions were analyzed as before. Purity was determined by SDS-PAGE. In order to obtain N-terminal amino acid sequence, 10 μg of the purified *N. gruberi* protease was loaded onto a 12% SDS gel and run at 20 milliamps for 1 hour. The gel was then subject to electroelution onto an immobilon filter (Millipore Corporation, Bedford, MA) as decribed previously (25). The blot was stained with

Coomassie blue and the 30 kD band was subject to gas-phase N-terminal protein sequencing (26).

pH profile. Purified enzyme was assayed for proteolytic activity using ZVLR-AMC as substrateas described above. The assays were carried out in a"universal" buffer, consisting of 10mM each of barbital buffer, sodium citrate, boric acid, and monobasic potassium phosphate (all from Sigma Chemical Co.) which was adjusted to the appropriate pH (ranging from pH 5.0-9.0) with sodium hydroxide. 2 mM DTT was added for all assays.

Effects of inhibitors. Inhibitors were preincubated with purified enzyme at the indicated concentrations shown in table 2 for 5 min at room temperature. Substrate (ZVLR-AMC) was then added and initial reaction velocity was measured immediately in the fluorimeter. All inhibitors were obtained from Sigma except E-64 and ZFA-fmk, which were obtained from Enzyme Systems Products. Stock solutions of all inhibitors were made up in DMSO. An equal volume of DMSO was tested with the enzyme, and inhibition relative to this control was calculated.

Assay of extracellular matrix degradation. The method for the preparation of extracellular matrix has been described in detail previously (11) Briefly, R22 cells (derived from rat vascular smooth muscle) were grown in 24-well Multiwell tissue culture plates (Becton Dickinson, Lincoln Park, NJ) in medium supplemented with [^{3}H]proline. Under these conditions, the cells produce a matrix of radioactively labeled glycoproteins, collagen and elastin. After the

cells were removed from the wells with NH_4OH, the insoluble matrix was washed and stored until used. Enzyme samples were added to the wells, and extracellular matrix degradation was assayed by the presence of released ^3H using a scintillation counter.

Assay of cytopathic effect. Baby hamster kidney (BHK) cells were grown to confluence in 24-well Multiwell tissue culture plates (Becton Dickinson) in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Cell Culture Facility, University of California, San Francisco). Whole trophozoites (10^5) or crude secretions (1 ml) were added to triplicate wells. ZFA-fmk (in DMSO) was added to some of the wells, and an equivalent amount of DMSO (to a final concentration of 1%, v/v) was added to other wells, to control for effects of solvent. The wells were observed hourly for disruption of the BHK cell monolayer and photographed.

Results

Demonstration of secreted protease activity. Secreted protease activity was first identified in cell-free growth media and phosphate buffered saline in which amebae had been incubating (conditioned media, conditioned PBS). Approximately 5 X 10^7 amebae were incubated in 30 mls of fresh media or PBS for 24-48 hours. The conditioned media/PBS was poured off and remaining amebae were spun away. The cell-free fluid was then assayed for the presence of protease activity using short peptide substrates to which a flourimetric leaving group (aminomethylcoumarin, AMC) was covalently bonded . Of the substrates tested, protease activity was found from both species using the substrate benzoxycarbonyl-valine-leucine-arginine-AMC (ZVLR-AMC). While ZVLR-AMC was found to be the best substrate, Z-phenylalanine-arginine-AMC (ZFR-AMC), and z-phenylalanine-proline-arginine-AMC (ZFPR-AMC) were also hydrolyzed by conditioned media from both species, in decreasing order of efficiency (data not shown). Secreted cysteine protease activity using the VLR-AMC assay was noted in 6 other *N. fowleri* strains tested (strains M75, C64, C61, 13B2M, 8B2M, CF1K, kindly provided by Dr. Harry Huizinga, Illinois State University) in roughly equivalent amounts (data not shown).

Purification of protease activity. Conditioned PBS from approximately 10^8 amebae was subjected to anion exchange chromatography (as described in materials and methods), and fractions obtained were assayed for ZVLR-AMC hydrolysis and SDS-

PAGE. The purification schemes are outlined in table 1. From both *N. fowleri* and *N. gruberi* 2 chromatographically distinct protease species were obtained. From *N. fowleri*, a significant proportion (70%) of the protease activity remained in the flow-through (void volume) despite runs on anion-exchange columns (Mono-Q and Mono-P) at relatively high pH (8.3) or on cation-exchange columns (Mono S) at relatively low pH (5.5). This species ran at a very high molecular weight (Mr=200 kD) on gelatin substrate SDS-PAGE, but ran at 28 kD (after boiling/reducing) on conventional SDS-PAGE (fig. 1A). The remaining activity (30% of total) which did bind to the anion-exchange columns migrated at a pI (pH of fraction in which peak activity was obtained) of 4.8, and on gelatin substrate SDS-PAGE demonstrated activity at 60 kD and 28 kD (fig 1A). The biochemical properties (to be outlined subsequently) of these two species of *N. fowleri* protease activity were otherwise indistinguishable.

N. gruberi protease activity was present in two peaks (each with 50% of the total recovered activity) from the Mono-P column. SDS-PAGE revealed a 30 kD species corresponding to a pI of 5.0, and a 27 kD species corresponding to a pI of 4.8. Gelatin substrate SDS-PAGE of each of these two species showed that each had protease activity at 60 kD and 27 kD (fig 1B). Again, the two species of *N. gruberi* protease (27 kD and 30 kD) were indistinguishable biochemically (to be outlined subsequently).

Characterization of protease activity. Protease activity was dependent on the presence of the reducing agent dithiothreitol (DTT)

in the assay buffer. In the absence of DTT, the rate of VLR-AMC hydrolysis was 0-10% that of activity in buffer containing 2mM DTT, which was optimal for the *N. fowleri* and *N. gruberi* protease. Activities at 5mM and 10mM were nearly equivelent to that in 2mM DTT (fig. 2). Protease activity from both species was maximally active at neutral pH (7.5) (fig. 3).

In order to further define the class of protease present, a panel of protease inhibitors was tested for their ability to inhibit activity (table 2). As shown in table 2, the cysteine protease inhibitor E64 completely abolished protease activity at 1 μM. N-ethyl-malemide (NEM), another cysteine protease inhibitor, did not inhibit activity at a concentration of 1 mM. While it is unclear why NEM failed to inhibit activity, E64 is known to be highly selective and more specific for cysteine proteases, reacting stoichiometrically to inhibit activity (35). Additionally, leupeptin, another cysteine protease inhibitor, inhibited activity at 10 μM (table 2). Serine (PMSF) and aspartic (pepstatin) protease inhibitors were not active against the enzyme (table 2). 1,10 phenanthroline (a metalloproteinase inhibitor) inhibited activity at 1 mM. However, EDTA, which also chelates ions necessary for metalloproteinase activity, did not inhibit activity at concentrations of 1 and 10 mM (data not shown). Although there are some inconsistencies, the inhibitor profile overall is most consistent with an enzyme of the cysteine class. The fact that the proteases are fully dependent on DTT (fig. 2) is a further argument favoring that it is a cysteine

protease, but sequence data was required to confirm this assumption.

To corroborate the biochemical evidence for a cysteine protease, N-terminal amino acid sequence of the *N. gruberi* protease was obtained as described in Materials and Methods. The sequence obtained was found to be 70% identical with that of chicken cathepsin L over 21 N-terminal residues, placing it within this subclass of cysteine proteases. A comparison of the sequence with other known cysteine proteases is shown in table 3.

Role of protease in pathogenesis of PAM. We next wished to assess the role of the secreted cysteine protease of *Naegleria* in the pathogenesis of PAM. We first demonstrated that a synthetic peptide, Z-phenylalanine-alanine fluoromethyl ketone (ZFA-fmk) inhibited protease activity from both species, completely abolishing it at a concentration of 1 μM (table 2). This peptide inhibitor inhibits cysteine protease activity via covalent modification of the active site cysteine residue of these enzymes but does not disrupt other cellular functions. No toxicity to mammalian cells is observed below 100 μM (19). This action of the inhibitor enabled us to test the effect of the inhibitor on whole amebae or ameba-free conditioned medium in assays of pathogenicity.

We first tested the ability of whole amebae and media conditioned by growing amebae to degrade extracellular matrix, a feature which would be essential for the tissue invasion which *Naegleria* exhibits.

Using an assay of extracellular matrix produced by vascular smooth muscle (R22) cells (10) we found that whole amebae or conditioned media degraded the matrix material, as assayed by release of tritiated matrix residues into solution. Significantly, ZFA-fmk inhibited proteolysis of the matrix material as shown in figure 4.

The amebae were cytopathic to cultured mammalian cells, and we demonstrated that this was due, at least in part, to cysteine protease activity. Whole amebae were placed over a monolayer of cultured mammalian (BHK) cells. After incubation for 4 hours, disruption of the monolayer occurred, as had been noted by other investigators previously (28). Significantly, addition of ZFA-fmk inhibited the ability of amebae to disrupt the monolayer. Representative photographs after a 4 hour incubation with and without inhibitor are shown in figure 5. Similar monolayer disruption was obtained using 1 ml of crude conditioned media (which contains the cysteine protease after trophozoites are removed). This was also inhibited by 100 μM ZFA-fmk (data not shown).

Discussion

We have shown that pathogenic and non-pathogenic species of *Naegleria* secrete a protease. Biochemical characterization, based on inhibitor studies and DTT-dependence indicate that it is a cysteine protease, and N-terminal sequence analysis shows that it belongs to the cathepsin L subclass of cysteine proteases. The proteases secreted by each species, quite similar to one another, are active at neutral pH. The protease was responsible for the degradation of extracellular matrix formed by R22 smooth muscle cells. It was also responsible for the production of a cytopathic effect on cultured mammalian cells. Qualitatively similar effects were observed whether whole amebae or amebae-free secretions were used. A specific cysteine protease inhibitor, ZFA-fmk, inhibited enzyme activity, extracellular matrix degradation, and cytopathogenicity.

The pathogenesis of PAM requires invasion through the nasal mucosa, cribiform plate and finally neural tissue. One might expect that a number of factors, including proteases and lipases would all be necessary for invasion through these tissue layers. A variety of cytopathic mechanisms by *Naegleria* have been described using either whole amebae or ameba lysates (11,28,29,30), and it is currently unclear whether direct ameba-cell contact or soluble cytolytic factors are the primary means of cytopathogenicity and tissue invasion, as both mechanisms have been proposed as important. Our work suggests that a secreted cysteine protease may

be a necessary component for the pathogenesis of PAM, and may be the active factor in amebic lysate assays of cytopathogenicity. This proposed role is not unlike that observed in previous studies of cysteine proteases in other parasitic diseases, most notably the pathogenic ameba *E. histolytica*. Both *Naegleria* and *E histolytica* secrete cysteine proteases, and both are thought to produce a pore-forming protein ('amebapore') which might play a role in cytopathogenicity. One hypothesis is that these are independent virulence factors involved in tissue invasion; another is that the pore-forming protein requires proteolytic activation by the cysteine protease (15).

We showed that the non-pathogenic species, *N. gruberi*, also secreted the cysteine protease. *N. gruberi* is incapable of proliferating well at temperatures above 30°C, and this alone may render it non-pathogenic. Alternatively, other virulence factors may be absent. However, we would hypothesize that if *N. gruberi* were adapted to growth at 37°C, it would be pathogenic. The protease itself was active at 37°C, suggesting that other metabolic factors are responsible for its temperature sensitivity.

HPLC of *N. fowleri* and *N. gruberi* proteases revealed two chromatographically distinct protease species from each. From the pathogenic strain, a species was obtained which did not bind to anion- or cation-exchange columns despite attempts at various buffer conditions. This species ran at very high Mr on gelatin substrate gels, but ran at 28 kD after boiling/reduction on

conventional SDS-PAGE. A possible explanation for this behavior is that the secreted protease exists as an aggregate with itself or other proteins which can be disrupted by boiling and disulfide reduction. That fraction of protease activity which did bind and elute from the anion-exchange columns may represent the protease monomer of this aggregate.

The protease activity from *N. gruberi* revealed two peaks, 30 kD and 27 kD, from the Mono-P column. Since no N-terminal 'leader' sequence was seen on N-terminal sequencing of the 30 kD species, it is reasonable to assume that the 27 kD species is a degradation product of its larger counterpart, either from artifactual autolytic degradation or via normal intracellular processing. The two species are identical on gelatin substrate SDS-PAGE, and appear similar on gelatin gels to the minor *N. fowleri* species, with bands at approximately 60kD and 30 kD. Of note is previous work with *E. histolytica*, in which cysteine protease activity has been demonstrated at similar molecular weights on gelatin substrate gels, at 56kD and 27 kD (14, 15).

A fluoromethyl ketone peptide inhibitor, ZFA-fmk, was shown to inhibit the protease, presumably through irreversible binding to the active site. The inhibitor has previously undergone animal trials as a potential therapeutic agent for rheumatoid arthritis and cancer metastasis. It was non-toxic at blood levels capable of producing enzyme inhibition and has an LD_{50} of 326 mg/kg in mice (24). The identification of a possible virulence factor and a relatively non-

toxic inhibitor presents a testable hypothesis that ZFA-fmk may attenuate the progression of PAM. Work is in progress to determine its usefulness as a possible therapeutic agent.

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Figure/Table legends (in order of appearance in text)

Table 1. Scheme of, and yields from, protein purification. Amebae conditioned PBS was subjected to anion exchange chromatography. Both species demonstrated two peaks of activity, which are added together in each part ofthis figure. A: *N. fowleri* protease; B: *N. gruberi* protease.

Figure 1. SDS-PAGE of HPLC partially-purified proteases (after Mono-Q, Mono-P columns). Standard and gelatin substrate gels were run as described in Materials and Methods. Fractions are named as outlined in Results. Molecular weight standards are as described. A: Partially purified *N. fowleri* protease. lane 1, major fraction; lane 2, minor fraction; lane 3, gelatin substrate gel of major fraction; lane 4, gelatin substrate gel of minor fraction. B: *N. gruberi* protease. lane 1, high molecular weight species; lane 2, low molecular weight species; lane 3, gelatin substrate gel of high molecular weight species; lane 4, gelatin substrate gel of low molecular weight species.

Figure 2. Dependence of protease activity on the presence on DTT. VLR-AMC assays were performed as described in Materials and Methods using HPLC-purified enzyme, varying the concentration of DTT. Results are expressed as percent of maximal activity. A: *N. fowleri*. B: *N. gruberi*.

Figure 3. pH optimum of purified proteases. VLR-AMC assays were performed as described in "universal" buffer (see Materials and Methods) with 2mM DTT. A: *N. fowleri.* B: *N. gruberi.*

Table 2. Inhibitory profile of secreted proteases. AMC assays were run as described, with a pre-incubation of inhibitor and enzyme for 5 min at room temperature. Stock solutions of inhibitors were in DMSO, and an equivelent amount of DMSO was added to the control tube.

Table 3. N-terminal sequence analysis of 30-kd species from *N. gruberi*, compared to sequence from other known known cysteine proteases.

Figure 4. Extracellular matrix degradation by intact amebae showing inhibition of degradation by ZFA-fmk. A, *N, fowleri*; B *N. gruberi.* In both cases curve a is with no ZFA-fmk; curve b is with ZFA-fmk added as described in Results. 1% DMSO was present in all samples.

Figure 5. Demonstration of cytopathic effect of whole amebae on mammalian cultured (BHK) cells. *N. fowleri* whole amebae were added over a monolayer of cultured cells, and incubated at 37 for 4 hours with and without ZFA-fmk. 1% DMSO was present in both samples. Effects were qualitatively similar for *N. gruberi*, and for condtioned media from both species. A, no ZFA-fmk. B, 100 uM ZFA-fmk.

Table 1A

Material	Amount total protein (mg)	Specific activity (fluor. units/mg)	-Fold purification	Total activity (units/s)	Percent recovery
Crude PBS	214.4	524.2	- -	112400	100
Mono Q peak	72.6	736.2	1.4	53450	47.6
Mono P peak	28.4	1085.7	2.1	30833	27.4

Table 1B

Material	Amount protein (mg)	Specific activity (fluor. units/mg)	-Fold purification	Total activity (units/s)	Percent recovery
Crude PBS	268.0.	478.5	- -	128242	100
Mono Q peak	37.2	1620.9	3.4	60310	47.0
Mono P peak	3.0	10026.4	20.9	30480	23.7

Figure 1A

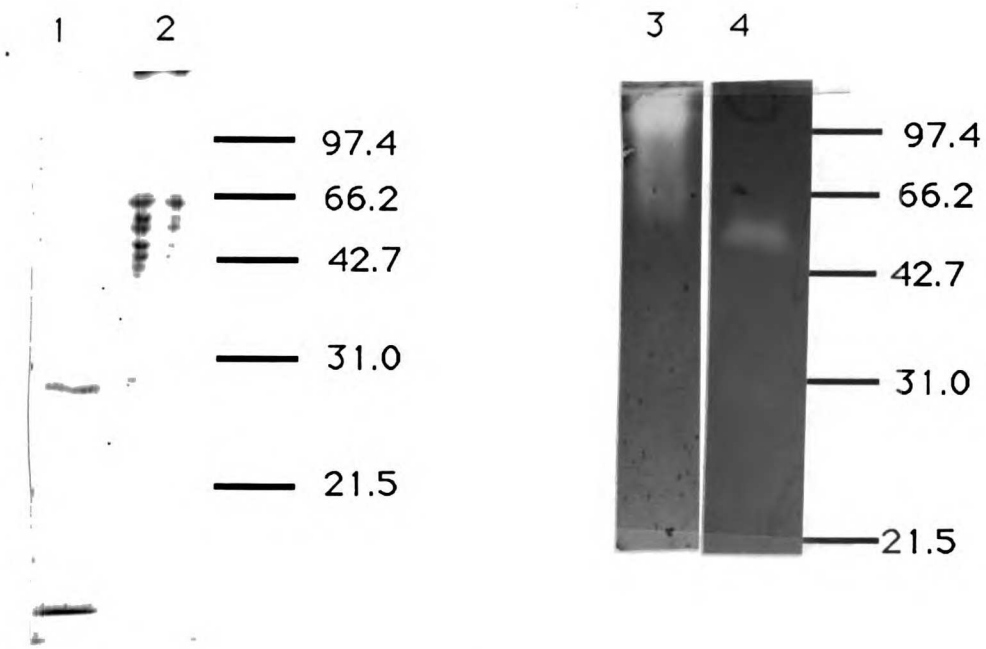


Figure 1B

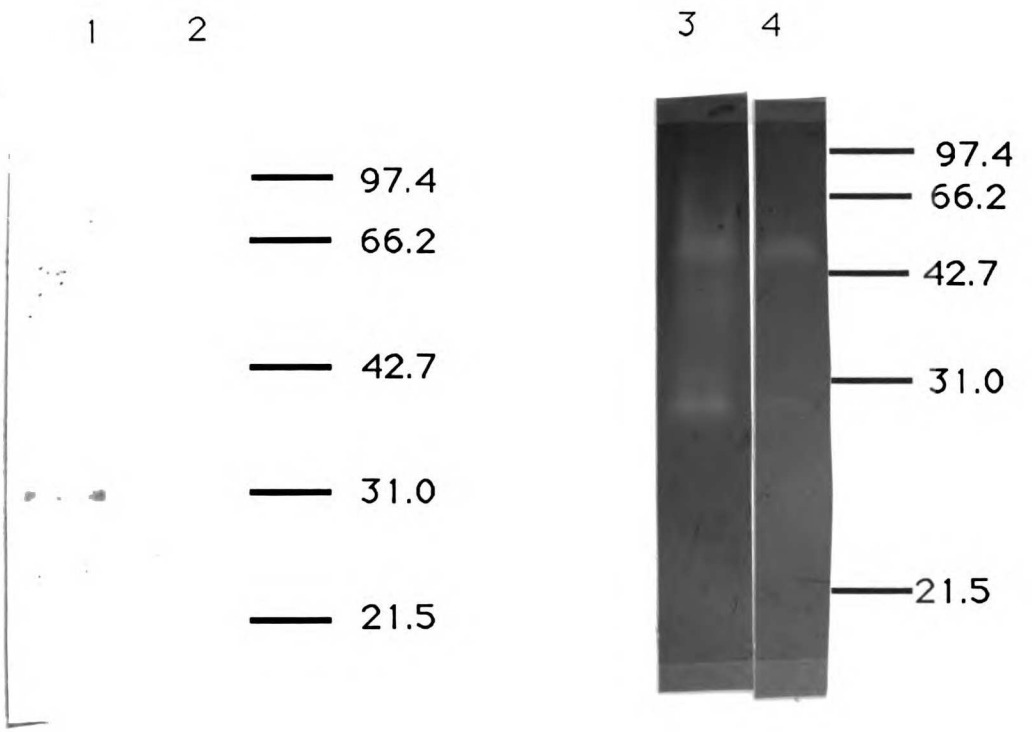


Figure 2A

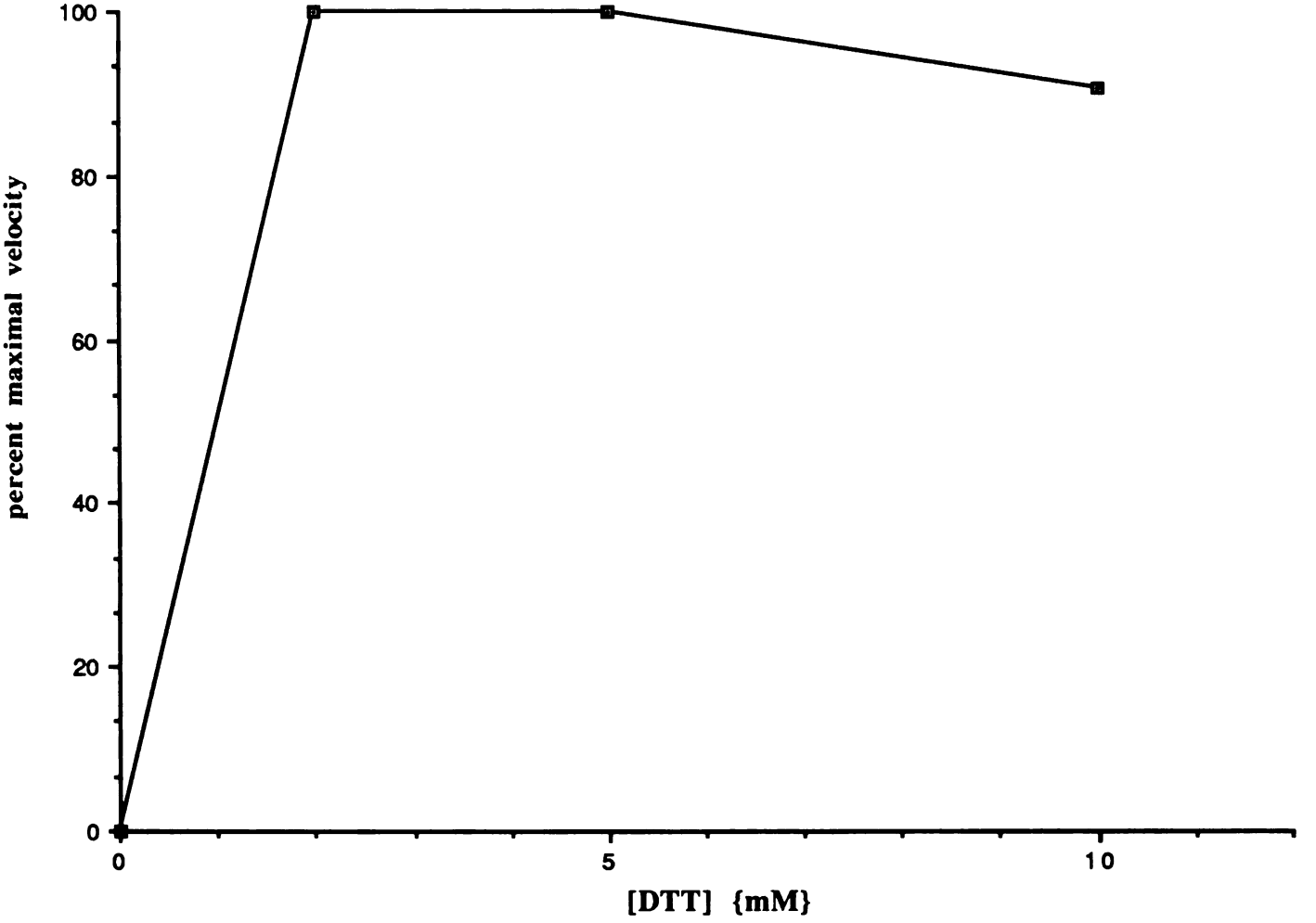


Figure 2B

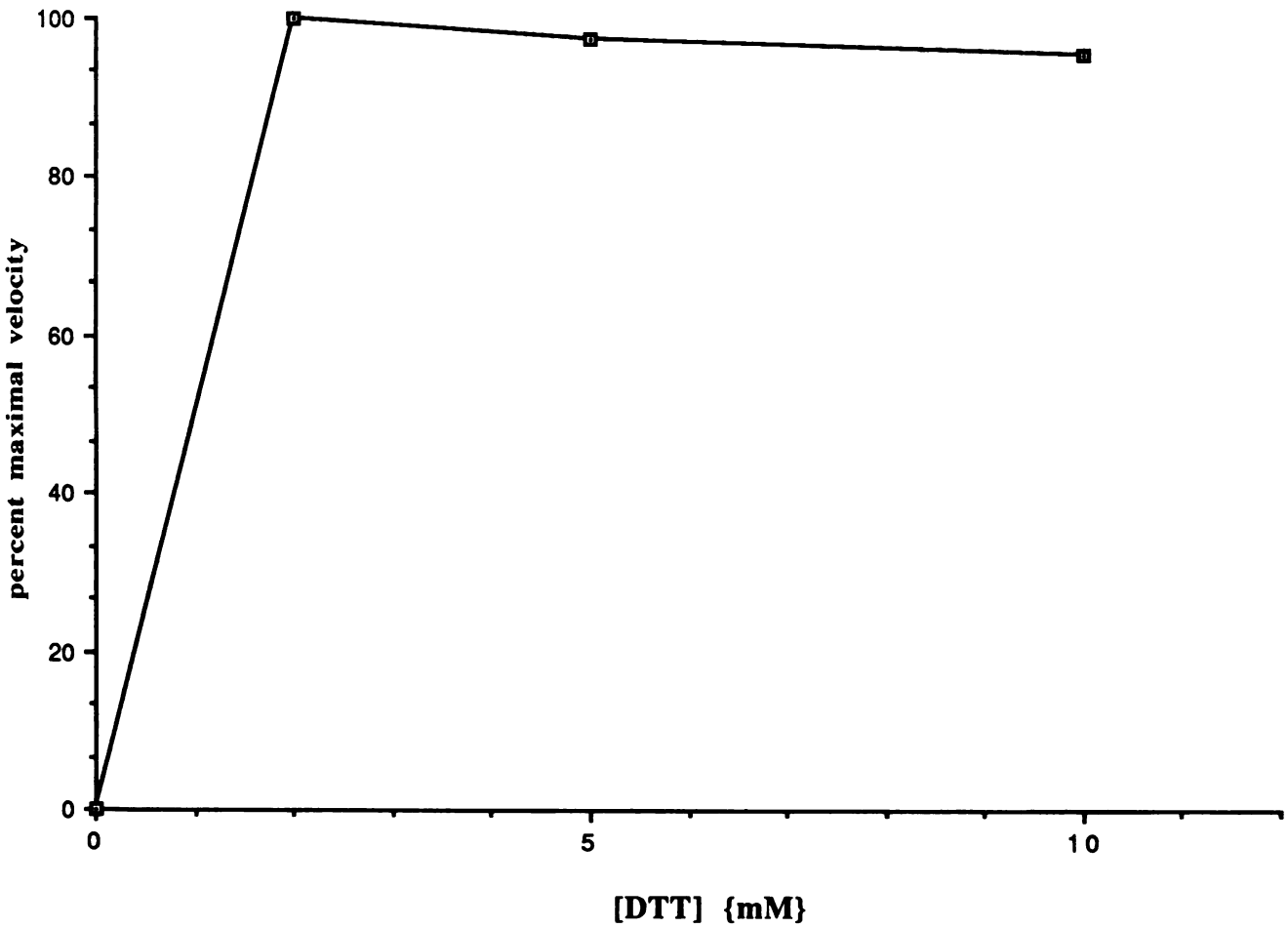


Figure 3A

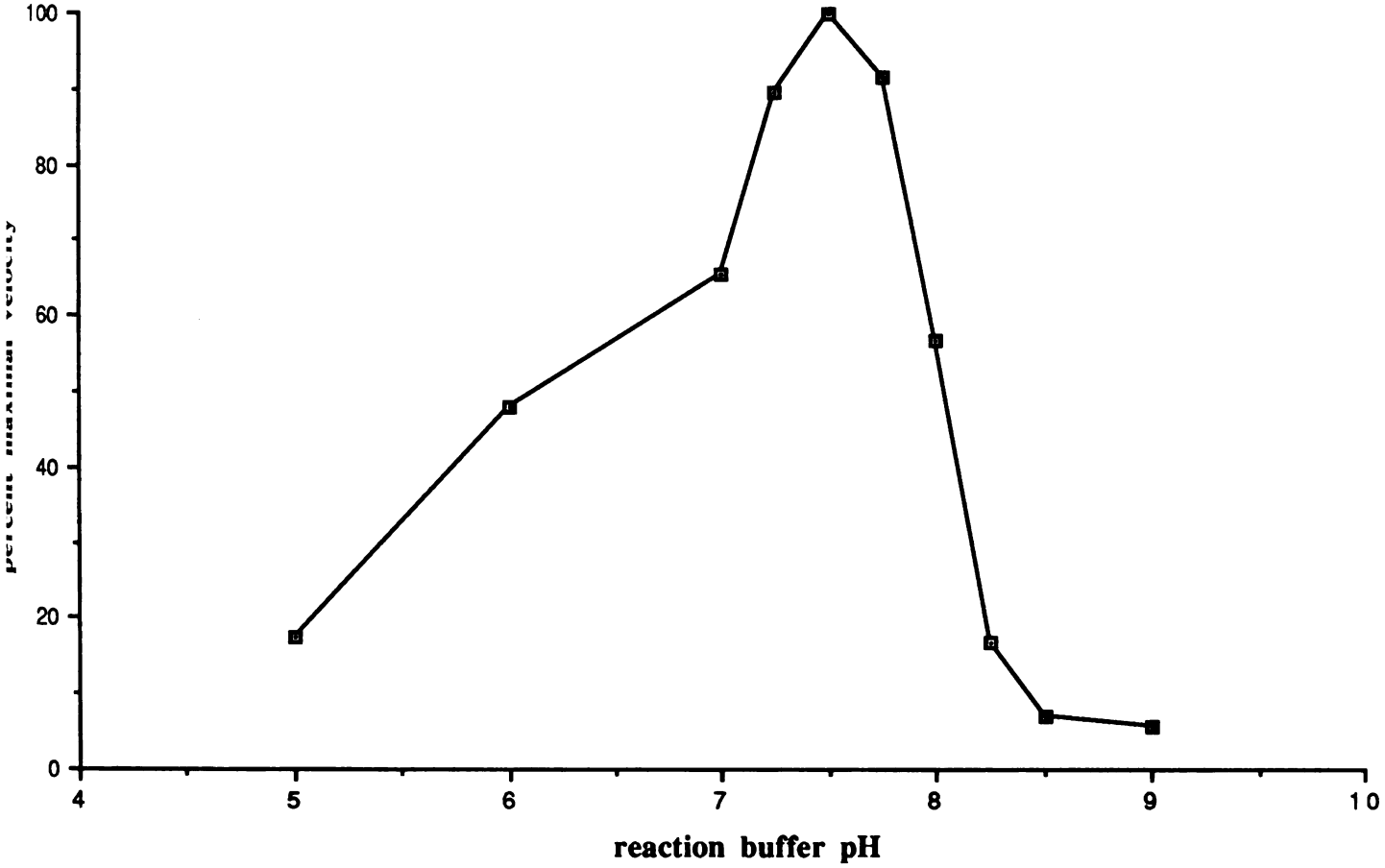


Figure 3B

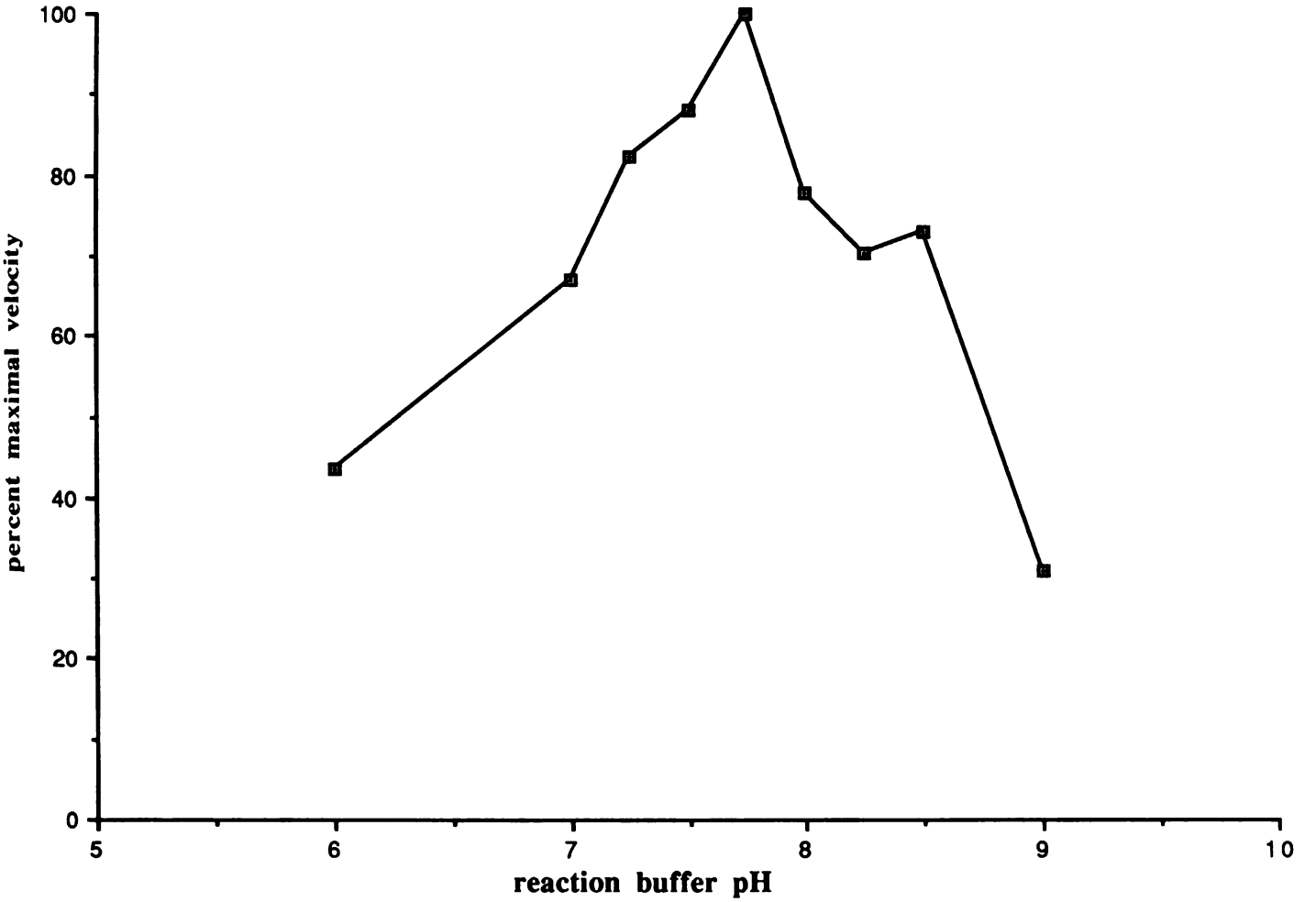

100
80
60
40
20
0
percent maximal velocity
5
6
7
8
9
10
reaction buffer pH

Table 2

Inhibitory profiles of purified proteins

Inhibitor	Percent inhibition of maximal velocity	
	N. fowleri	*N. gruberi*
ZFA-fmk (1μM)	100	100
E64 (1μM)	100	100
leupeptin (10μM)	100	100
NEM (1mM)	0	0
pepstatin (1mM)	0	0
1,10 phenanthroline (1mM)	73	69
PMSF (1mM)	0	0

Table 3

N-terminal sequence analysis of purified proteins.

Protease	Sequence	Identity
N. gruberi	APKEFDWREHNAVTPVKDQGN	--
chicken cathepsin L	APRSVDWREKGYVTPVKDQGI	70%
Dictyostelium cathepsin L	NPKSIDWRTKNAVTPIKDQ	70%
Papain	IPEYVDWRQKGAVTPVKNQG	67%
E. histolytica	APESVDWRSIMN--PAKNQG	50%

Figure 4A

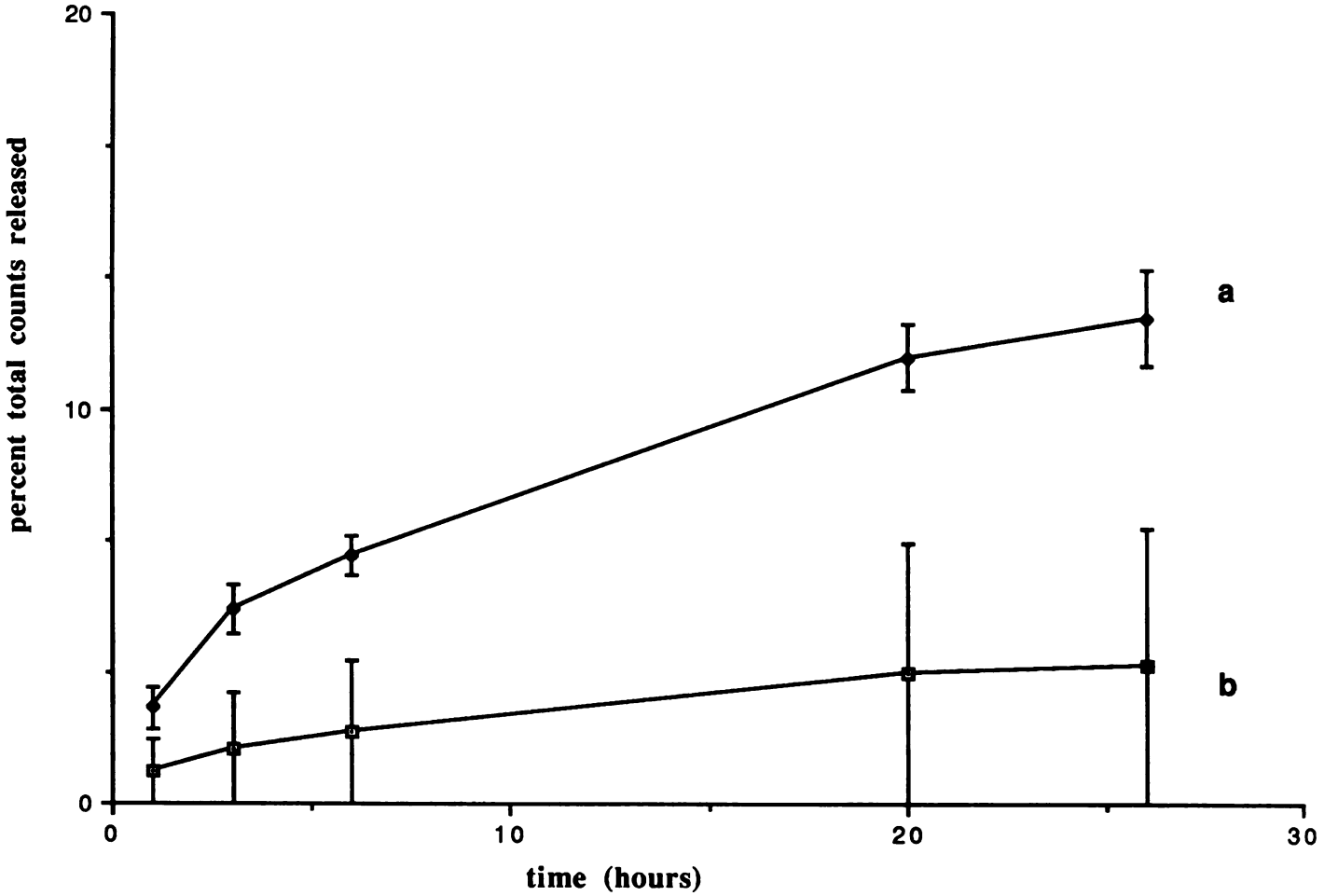


Figure 4B

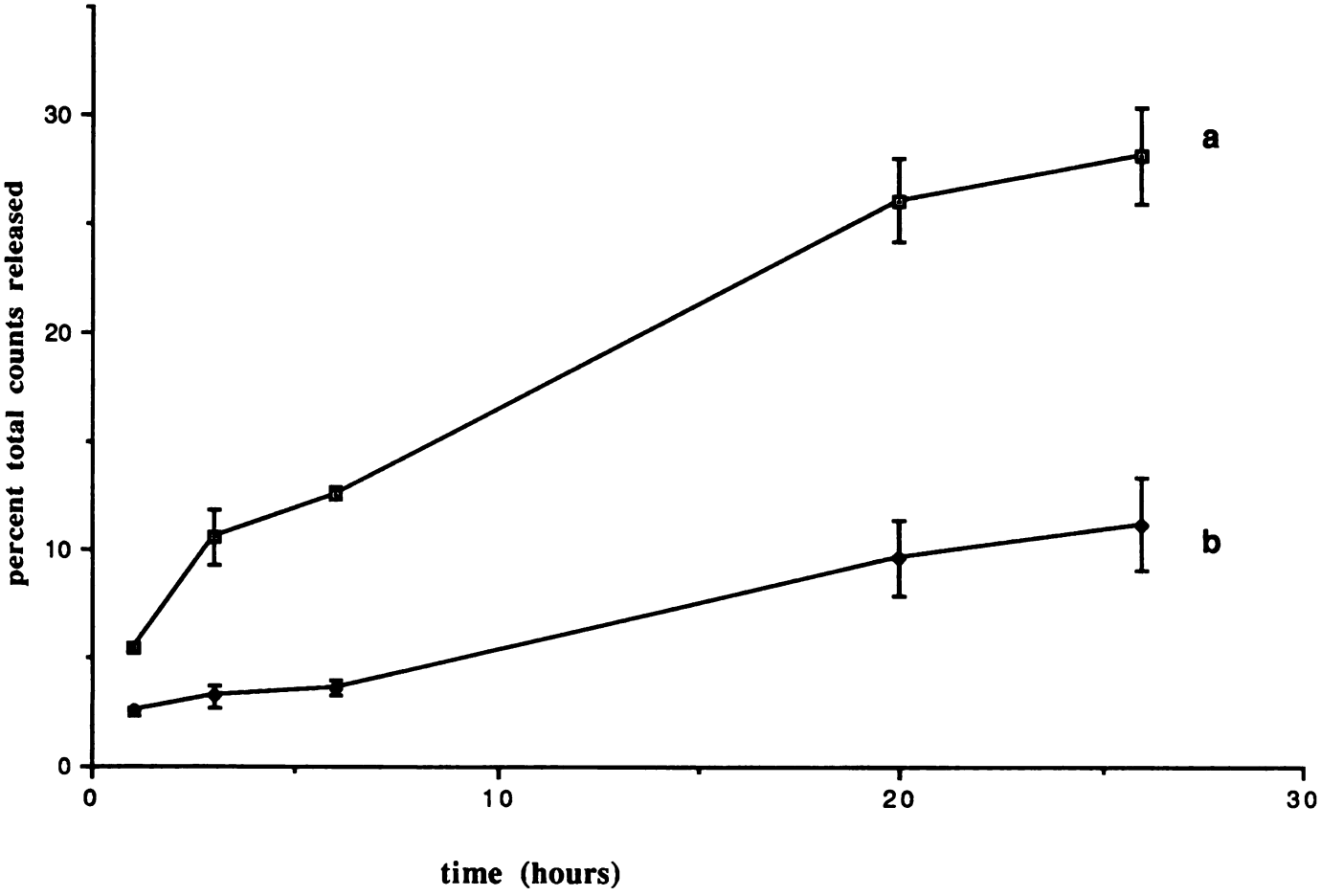
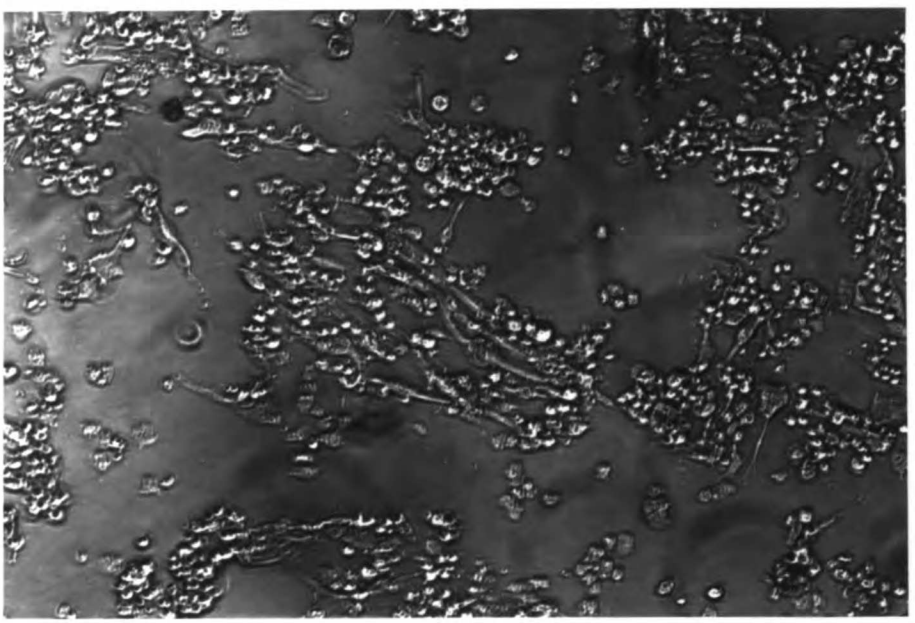
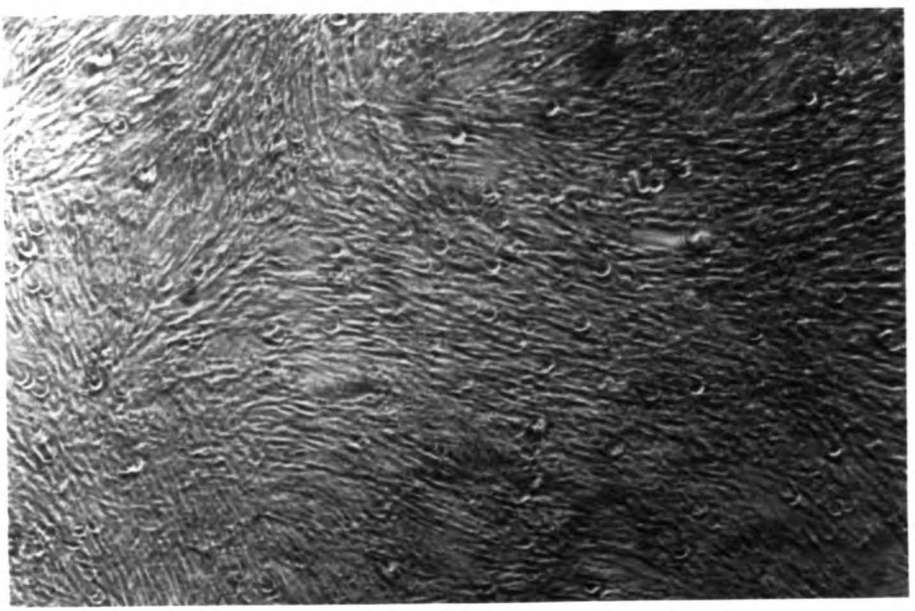

percent total counts released
30
20
10
0
0
10
20
30
time (hours)
a
b

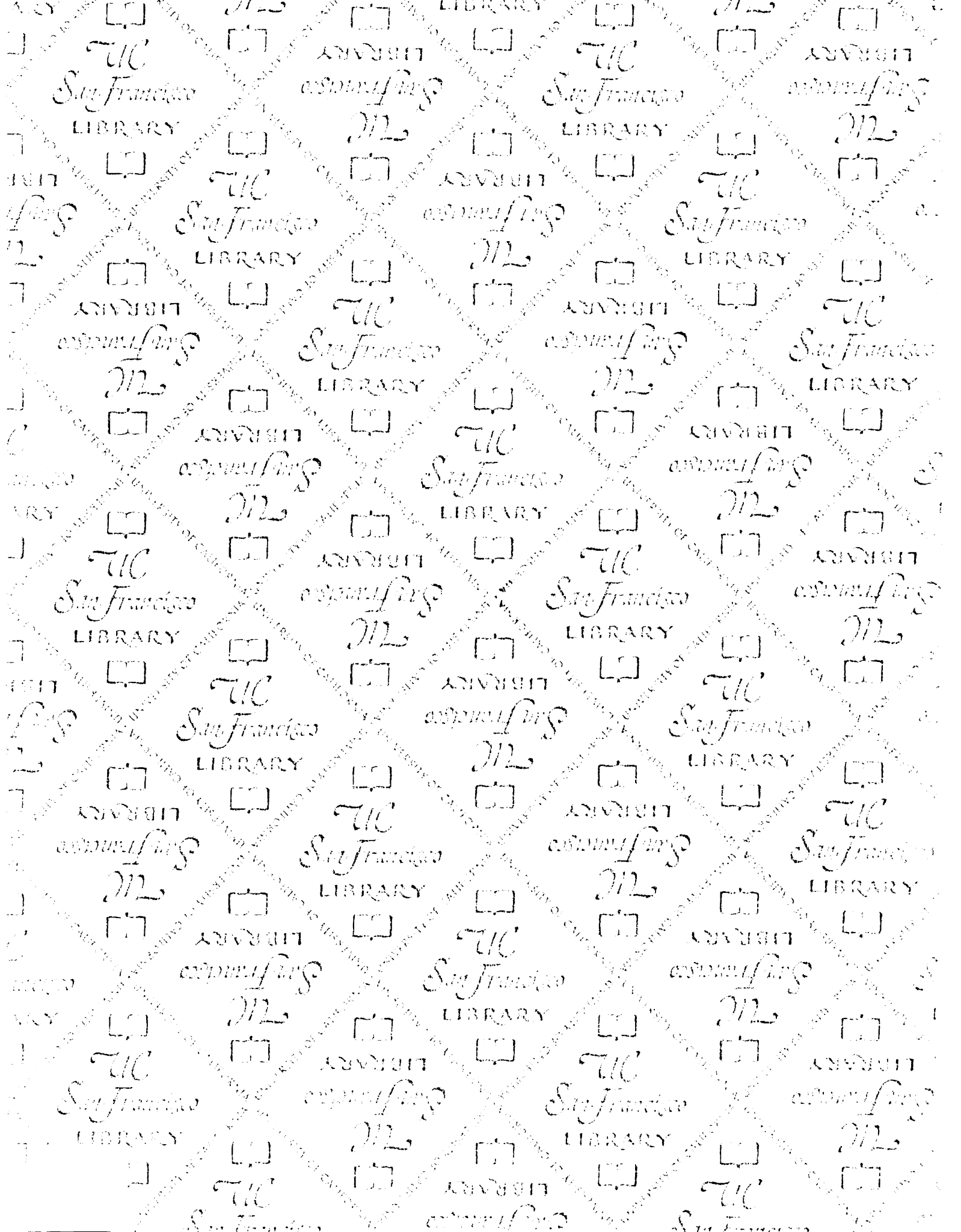
Figure 5

A



B

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