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Purification and Characterization of a Serine Proteinase from *Acanthamoeba culbertsoni*

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Abstract: A serine proteinase was purified from *Acanthamoeba culbertsoni* by 41~80% ammonium sulfate fractionation, ion exchange chromatography, affinity chromatography and gel filtration chromatography. The molecular weight of the purified enzyme was estimated to be 108.0 kDa by gel filtration chromatography and 54.0 kDa by SDS-PAGE. Therefore, the purified enzyme seemed to be a dimer. Isoelectric point was 4.5. The enzyme activity was highly inhibited by the serine proteinase inhibitors diisopropyl fluorophosphate (DFP) and phenylmethyl sulfonylfluoride (PMSF). It had a narrow pH optimum of 6.5~7.5 with a maximum at pH 7.0. These data suggested that the purified enzyme was a neutral serine proteinase. Optimal temperature was 37℃. It was stable for at least 16 h at 4℃ and 37℃, but it was rapidly inactivated at 65℃. The activity of the purified enzyme was not influenced significantly by Mg^{2+}, Mn^{2+}, Zn^{2+} or Ca^{2+}. However, the enzyme activity was highly inhibited by Hg^{2+}. The enzyme degraded type I collagen and fibronectin, but not BSA, hemoglobin, lysozyme, immunoglobulin A or immunoglobulin G.
Key words: *Acanthamoeba culbertsoni*, characterization, purification, serine proteinase.

Small, free-living amoebas have been regarded as exotic organisms encountered in rare infections such as meningoencephalitis and keratitis. Pathogenic, free-living amoebas may enter the nasal cavity by inhalation of dust or aspiration of water contaminated with trophozoites or cysts (Lawanda, 1979). Inhalation or aspiration of aerosols containing the cysts is another possible source.

Respiratory symptoms in some patients may be the result of hypersensitivity or of subclinical infection with free-living amoebas. Chronic ulceration of the skin containing amoebic trophozoites and cysts has been reported in patients who have died of granulomatous amoebic encephalitis (GAE). These cases may represent a terminal hematogenous dissemination of amoebic trophozoites and cysts from primary foci in the lungs or from the central nervous system (CNS) rather than a primary dermatologic lesion (Martinez, 1985).

Acanthamoeba can directly infect the cornea generally after trauma associated with contaminated water or contact lens wear, causing extremely serious keratitis (Jones, 1986). During the course of amoeba host tissue invasion and multiplication in various body sites such as the olfactory mucosa and the brain, there is evidence of extensive tissue destruction. Pathologic studies of involved cornea in an early case showed destruction of the anterior cornea, with infiltration of acute inflammatory cells into the superficial and middle layers of the corneal stroma (Theodore *et al.*, 1985). When penetrating keratoplastics were performed at a later stage in the disease process, considerable loss of corneal substance had occurred and was followed by ulceration, descemetocele formation and finally perforation of the cornea (Ma *et al.*, 1990).

Until recently it has been assumed that inflammatory cells, principally neutrophils, were responsible for the corneal pathology. However, the mediators of tissue injury are believed to be of both amoebic and host origin (Ferrante and Thong, 1983; Brooks and Schneider, 1985; Dunnebacke and Trowan-Kelly, 1985). For example, amoebas are purported to cause tissue damage by releasing cytopathogenic substances, oxygen radicals, and a number of different enzymes. Penetration of mucosal surfaces by these amoebas most likely depends on the capacity to damage tissue.

Parasite proteinases are important in tissue invasion, migration, and host pathology (McKerrow, 1987). Some evidence now suggests a role for parasite proteinases in acanthamoebal keratitis, but its presence is not an indicator of amoeba virulence. Proteinase activity has been reported from a number of protozoan parasites (McKerrow *et al.*, 1993), including parasitic amoebas, and much of the pathology observed with *Entamoeba*

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histolytica infections is related to secreted and intracellular proteinases (Gitler *et al.*, 1984; Lushbauch *et al.*, 1984). A recent study reported that the excretory and secretory (E & S) products from trophozoites of *Acanthamoeba castellanii* induced damage to collagen shields in an *in vitro* assay and *in vivo* in rat corneas (He *et al.*, 1990). Furthermore, the proteinase of *Acanthamoeba polyphaga* had nonspecific collagenolytic activity which was thought to be closely associated with its pathogenicity (Mitro *et al.*, 1994). Therefore, an amoebic proteinase could participate in destruction of host-protective barriers and promote amoeba tissue invasion and destruction. However, detailed investigation has been hampered by the lack of purified proteinase.

In this study we aimed to purify and characterize proteinase from trophozoites of *Acanthamoeba culbertsoni* and partially investigate its role associated with acanthamoebiasis.

Materials and Methods

Culture and harvest

Acanthamoeba culbertsoni was cultured in CGV medium (Willaert and Leray, 1973) at 37℃ for 7~8 days. The cells were harvested by centrifugation at 3,000 rpm for 30 min and washed several times with cold saline solution and preserved at −70℃ until used.

Preparation of crude extracts

Harvested cells were sonicated with ultrasonicator (Ultrasonic processor, Ultrasonics, Inc.) in 0.1 M phosphate buffered saline (pH 7.2) for three times, 15 sec each, on the ice and centrifuged at 15,000 rpm at 4℃ for 60 min. The supernatants were collected and subjected to further purification.

Assay of enzyme activity

Proteinase activity was estimated by synthetic dipeptide substrate with a fluorescent leaving group, carboxybenzoyl-arginine-arginine-7-amino-4-trifluoromethylcoumarin (CBZ-arg-arg-AFC; Enzyme system products, USA). The assay mixture contained 10 μg of substrate in 0.1 M Tris-HCl buffer (pH 7.0) in a total volume of 0.5 ml. After incubation at 37℃ for 2 h with enzyme solution, the 7-amino-4-trifluoromethylcoumarin (AFC) liberated from the fluorogenic substrate was quantified with excitation at 400 nm and emission at 505 nm using a fluorometer (Sequoia-Turner Co. Model III, USA). One unit of the enzyme activity was defined as nmols of AFC produced per min under the reaction condition. Protein concentration was determined by the method of Lowry *et al.* (1951) with BSA (Sigma, USA) as standard.

Purification of enzyme

Step 1. Ammonium sulfate fractionation: The crude extract was fractionated with 41~80% ammonium sulfate. The precipitate was collected by centrifugation at 15,000 rpm for 1 h, dissolved in about 20 ml of 40 mM Tris-HCl buffer (pH 7.0), and dialyzed for 24 h against the same buffer of 2 L.

Step 2. DEAE Sepharose CL-6B ion exchange chromatography: The dialysate was applied onto a column (2.6×10 cm) of DEAE Sepharose CL-6B equilibrated with 40 mM Tris-HCl buffer (pH 7.0) and washed with the same buffer at a flow rate of 40 ml/h. Adsorbed proteins were eluted using a linear gradient of 0.0~1.0 M NaCl. The active fractions were pooled, dialyzed against distilled water at 4℃ and lyophilized.

Step 3. CM Sepharose CL-6B ion exchange chromatography: The lyophilized preparation was resolved in 70 mM Tris-HCl buffer (pH 6.8) and applied onto a column (2.6×10 cm) of CM Sepharose CL-6B equilibrated with same buffer. Adsorbed proteins were eluted using a linear gradient of 0.0~1.0 M NaCl. The active fractions were pooled, dialyzed against distilled water at 4℃ and lyophilized.

Step 4. Benzamidine Sepharose 6B affinity chromatography: The lyophilized preparation was resolved in 50 mM Tris-HCl buffer (pH 8.0) and applied onto a column (1.6×5 cm) of Benzamidine Sepharose 6B equilibrated with the same buffer. Adsorbed proteins were eluted with 1 M NaCl in the same buffer. The active fractions were pooled, dialyzed against distilled water at 4℃ and lyophilized.

Step 5. Sephacryl S-200 HR molecular sieve chromatography: Partially purified enzyme solution was dialyzed against 0.1 M Tris-HCl buffer (pH 7.0) and applied onto a column (1.6×100 cm) of Sephacryl S-200 HR equilibrated with the same buffer. Fractions (2.5 ml) of the eluate were collected and dialyzed against distilled water followed by lyophilization. The lyophilized powder was stored at −80℃ until used.

SDS-PAGE

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was performed in 7.5~15% (w/v) gradient slab gel as described by Laemmli (1970). The gels were stained with 0.1% Coomassie Brilliant Blue.

Determination of molecular weight

The molecular weight of the purified enzyme was determined by SDS-PAGE. The standard proteins were albumin bovine (66,000), albumin egg (45,000), glyceraldehyde 3-phosphate dehydrogenase (36,000), carbonic anhydrase (29,000), trypsinogen (24,000), soy bean

trypsin inhibitor (20,100) and α-lactalbumin (14,400) (Sigma, USA). The native molecular weight of the purified enzyme was determined by molecular sieve chromatography. The purified enzyme was applied onto a column (1.6×100 cm) of Sephacryl S-200 HR precalibrated with molecular weight standards. Standard proteins were aldolase (158,000), bovine serum albumin (67,000), ovalbumin (43,000) and chymotrypsinogen A (25,000).

Effect of proteinase inhibitors and metal ions on enzyme activity

The effect of proteinase inhibitors on enzyme activity was examined. The purified enzyme was preincubated at 37℃ for 30 min in 0.1 M Tris-HCl buffer (pH 7.0) containing inhibitors. Substrate was then added. The reaction mixtures were incubated at 37℃ for 2 h and enzyme activity was measured. Inhibitors used in this study were diisopropyl fluorophosphate (DFP, 100 μM), phenylmethyl sulfonylfluoride (PMSF, 1 mM), L-*trans*-epoxy-succinyl-leucylamido-(4-guanidino)-butane (E-64, 10 μM), leupeptin (100 μM), pepstatin A (1 μM), N-α-p-tosyl-L-lysine-chloromethyl ketone (TLCK, 100 μM), N-tosyl-L-phenylalanine-chloromethyl ketone (TPCK, 100 μM), iodoacetic acid (1 mM), 1,10-phenanthroline (1 mM) and ethylenediaminetetraacetic acid (EDTA, 10 mM). All inhibitors were purchased from Sigma Chemical Co. (St. Louis, USA). The effect of metal ions on enzyme activity was examined by the following method; The purified enzyme was incubated in 0.1 M Tris-HCl buffer (pH 7.0) containing 2 mM $MgCl_2$, $MnSO_4$, $CaCl_2$, $ZnCl_2$, and $HgCl_2$ at 37℃ for 2 h. Then each enzyme activity was measured.

Determination of isoelectric point

Isoelectric focusing was carried out on a Phast gel IEF (Pharmacia, Phast system, pH 3.0~9.0) and standard protein kit was used. After electrofocusing, the gels were stained with 0.1% Coomassie Brilliant Blue.

Substrate specificity

Substrate specificity of purified enzyme was assessed by using the various substrates including type I collagen, fibronectin, lysozyme, hemoglobin, BSA, immunoglobulin G (IgG) and immunoglobulin A (IgA). All the substrates were perchased from Sigma Chemical Co. and dissolved in 0.1 M Tris-HCl (pH 7.0) at the concentration of 1 mg/ml. Rabbit corneal extract was prepared by homogenizing the cornea of New Zealand white rabbit followed by centrifugation at 10,000 rpm for 30 min. The supernatant was collected and used. The purified enzyme (10 μg) was added to 90 μl of substrate solutions. The mixtures were incubated at 37℃ and aliquots (20 μl) were withdrawn from the reaction tube at increasing time intervals. The reactions were stopped by adding an equal volume of denaturing sample buffer (0.125 M Tris-HCl, pH 6.8, 2% SDS, 2% sucrose, 0.1% β-mercaptoethanol) followed by boiling the sample for 2 min. SDS-PAGE was performed with the method of Laemmli (1970).

Results

Purification of proteinase

A proteinase was purified from *Acanthamoeba culbertsoni* by a combination of 41~80% ammonium sulfate precipitation and four chromatographic procedures. The protein precipitate obtained by ammonium sulfate precipitation was applied to a DEAE Sepharose column. The active fractions (48~55 fractions) were pooled, concentrated and applied to a CM Sepharose column (Fig. 1A). The active fractions (7~9 fractions) were pooled, concentrated and applied to Benzamidine Sepharose column. Adsorbed proteins were eluted with 1 M NaCl in the same buffer. The enzyme purified partially was applied to Sephacryl S-200 HR column (Fig. 1B). The purified enzyme (fraction 37) migrated as a single band on SDS-PAGE of which the molecular weight was approximately 54.0 kDa (Fig. 2). However, the molecular weight of the native enzyme was estimated to be 108.0 kDa by Sephacryl S-200 HR gel filtration chromatography (Fig. 3). Therefore, the purified enzyme seemed to be a dimer. Isoelectric point was 4.5 (data not shown). The purification procedures are summarized in Table 1. The purification was 332.4 fold and yield was 11.5%.

Effect of inhibitors and metal ions

The effect of inhibitors on the activity of purified enzyme was determined. The enzyme activity was significantly inhibited by serine proteinase inhibitors, DFP and PMSF. Other inhibitors did not influence the enzyme activity (Table 2). The effect of metal ions on enzyme activity was also investigated. The enzyme activity was strongly inhibited by Hg^{2+}. While the activity was not inhibited by Ca^{2+}, Mn^{2+}, Zn^{2+} or Mg^{2+} (data not shown). However, there was no indication of sulfhydryl group involvement because no effect was observed by iodoacetic acid. These results suggested that the purified enzyme was a serine proteinase.

Effect of pH and temperature

The enzyme was active over a narrow pH range of 6.5 to 7.5 with a pH optimum at 7.0. The activity decreased gradually below pH 6.0 and above pH 8.0 (Fig. 4). Thus, the enzyme has a neutral optimal pH.

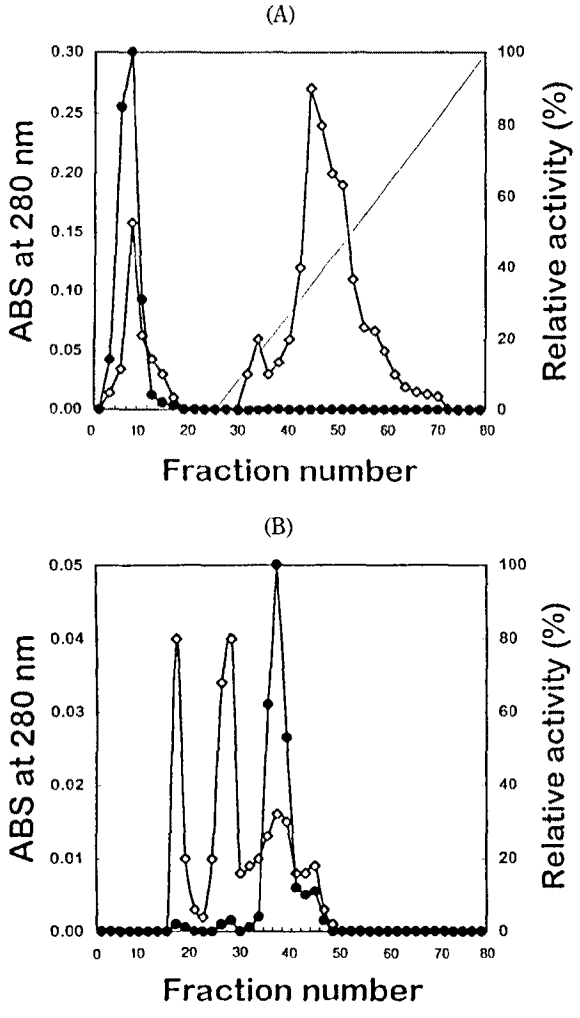

Fig. 1. (A) Chromatogram of the CM Sepharose CL-6B ion exchange chromatography. Linear gradient elution using 0~1 M NaCl (——), proteinase activity (●) and protein content at 280 nm (◇). (B) Chromatogram of the Sephacryl S-200 HR molecular sieve chromatography. Proteinase activity (●) and protein content at 280 nm (◇).

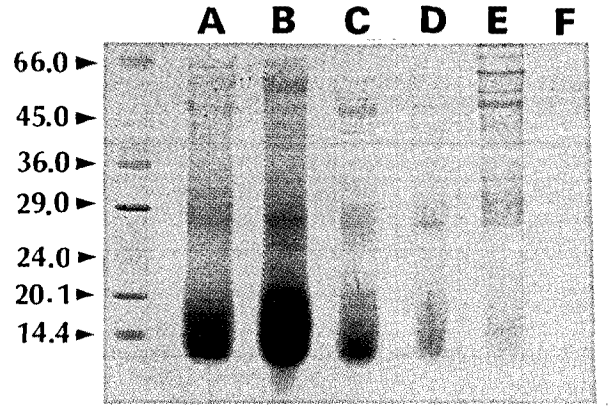

Fig. 2. Gradient SDS-PAGE (7.5~15.0%) analysis of the proteins in each step of the purification. Lane A, homogenate supernatant; lane B, ammonium sulfate precipitation (41~80%); lane C, active peak from DEAE Sepharose CL-6B; lane D, active peak from CM Sepharose CL-6B; lane E, active peak from Benzamidine Sepharose 6B; lane F, active peak from Sephacryl S-200 HR.

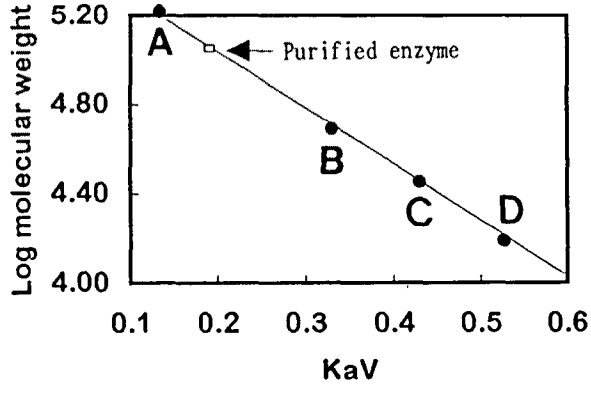

Fig. 3. Estimation of the molecular weight of the native purified enzyme by gel filtration chromatography. The molecular size marker proteins were as follow: A, aldolase (158,000); B, bovine serum albumin (67,000); C, ovalbumin (43,000); D, chymotrypsinogen A (25,000).

Table 1. Purification of serine proteinase from *A. culbertsoni*

Purification step	Total protein (mg)	Total activity[a] (U)	Specific activity[a] (U/mg)	Purification fold	Yield (%)
Crude extract	462.6	5510	11.9	1	100
ASP[b]	106.7	5490	51.5	4.3	99.6
DEAE[c]	38.4	4201	109.5	9.2	76.2
CM[d]	11.6	4038	349.6	29.4	73.3
Affinity[e]	4.9	3773	778.0	65.4	68.5
Sephacryl[f]	0.2	633	3956.0	332.4	11.5

[a] nmol of AFC production per min under reaction condition.
[b] Ammonium sulfate precipitation.
[c] DEAE Sepharose CL-6B ion exchange chromatography.
[d] CM Sepharose CL-6B ion exchange chromatography.
[e] Benzamidine Sepharose 6B affinity chromatography.
[f] Sephacryl S-200 HR gel filtration chromatography.

Table 2. Effect of inhibitors on the purified enzyme

Inhibitors	Relative activity (%)[b]
Control[a]	100.0
Cysteine class	
E-64 (10 μM)	107.5
Iodoacetic acid (1 mM)	105.1
Cysteine and serine class	
Leupeptin (100 μM)	129.4
TPCK (100 μM)	85.8
TLCK (100 μM)	91.2
Serine class	
DFP (100 μM)	3.4
PMSF (1 mM)	7.5
Aspartic class	
Pepstatin A (1 μM)	137.2
Metallo class	
EDTA (10 mM)	87.6
1,10-phenanthroline (1 mM)	93.0

[a]Control represents the activity tested without any inhibitors.
[b]% of the control.

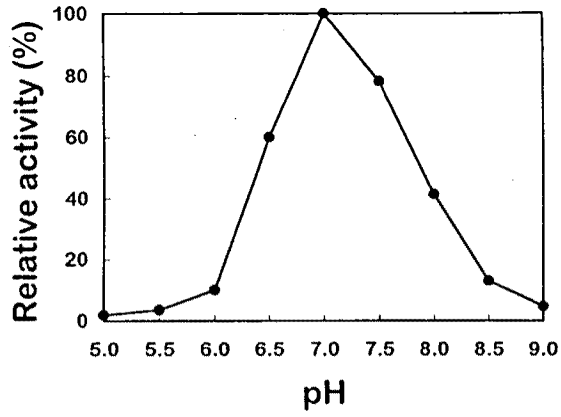

Fig. 4. Effect of pH on the activity of the purified enzyme. The enzyme activity was measured in 0.1 M sodium acetate buffer (pH 5.0~6.0), 0.1 M sodium phosphate buffer (pH 6.0~7.0), 0.1 M Tris-HCl buffer (pH 7.0~9.0). Maximal activity was shown as 100%.

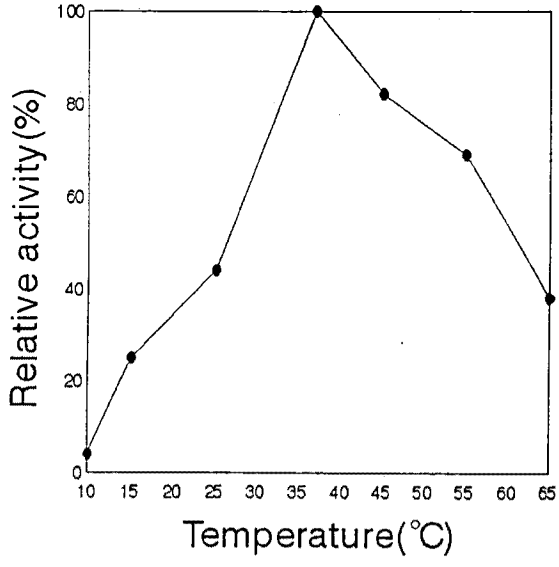

Fig. 5. Effect of temperature on the activity of purified enzyme. The purified enzyme was incubated at diffferent temperature from 10℃ to 60℃ for 2 h and the enzyme activities were measured. Maximal activity was shown as 100%.

The enzyme exhibited a broad temperature optimum with a maximum activity at 37℃ and was gradually inactivated above 45℃ (Fig. 5). The enzyme maintained its activity up to 80% at 4℃ and 37℃ for 16 h, but the activity was completely lost after one week and 120 h, respectively. It was highly unstable at 65℃ (data not shown).

Substrate specificity

Substrate specificity of the purified enzyme was assessed by using various substrates including type I collagen, fibronectin, lysozyme, hemoglobin, BSA, immunoglobulin G (IgG) and immunoglobulin A (IgA). The enzyme could degrade type I collagen. The original α-chain and β-chain of type I collagen were degraded by the enzyme and the amount of products increased in a time-dependent manner. Fibronectin was also degraded to many fragments by the enzyme. Rabbit corneal extract was degraded by the enzyme. However, lysozyme, hemoglobin, BSA, IgG and IgA were not degraded by the enzyme (Fig. 6). Therefore, the purified enzyme showed a narrow substrate specificity towards tissue constituent proteins such as collagen and fibronectin.

Discussion

Much of the pathology observed in pathogenic free-living amoeba infections is related to secretory and intracellular proteinases (Keene *et al.*, 1986; Montfort *et al.*, 1987; Scholze and Schulte, 1989; Aldape *et al.*, 1994). The most extensive studies of amoebal proteinases have been done on *Entamoeba histolytica*. The major intracellular and extracellular cysteine proteinase of *E. histolytica* has a pH optimum between 6 and 7, depends on reducing agents, is inhibited by thiol proteinase inhibitors, and exhibits multiple isoforms ranging in M_r from 16,000 to 66,000 (Keene *et al.*, 1986; Luaces and Barrett, 1988). A second proteolytic enzyme proposed to play an important role in the invasion of virulent *E. histolytica* trophozoites is a surface-associated or membrane-associated metallocollagen-

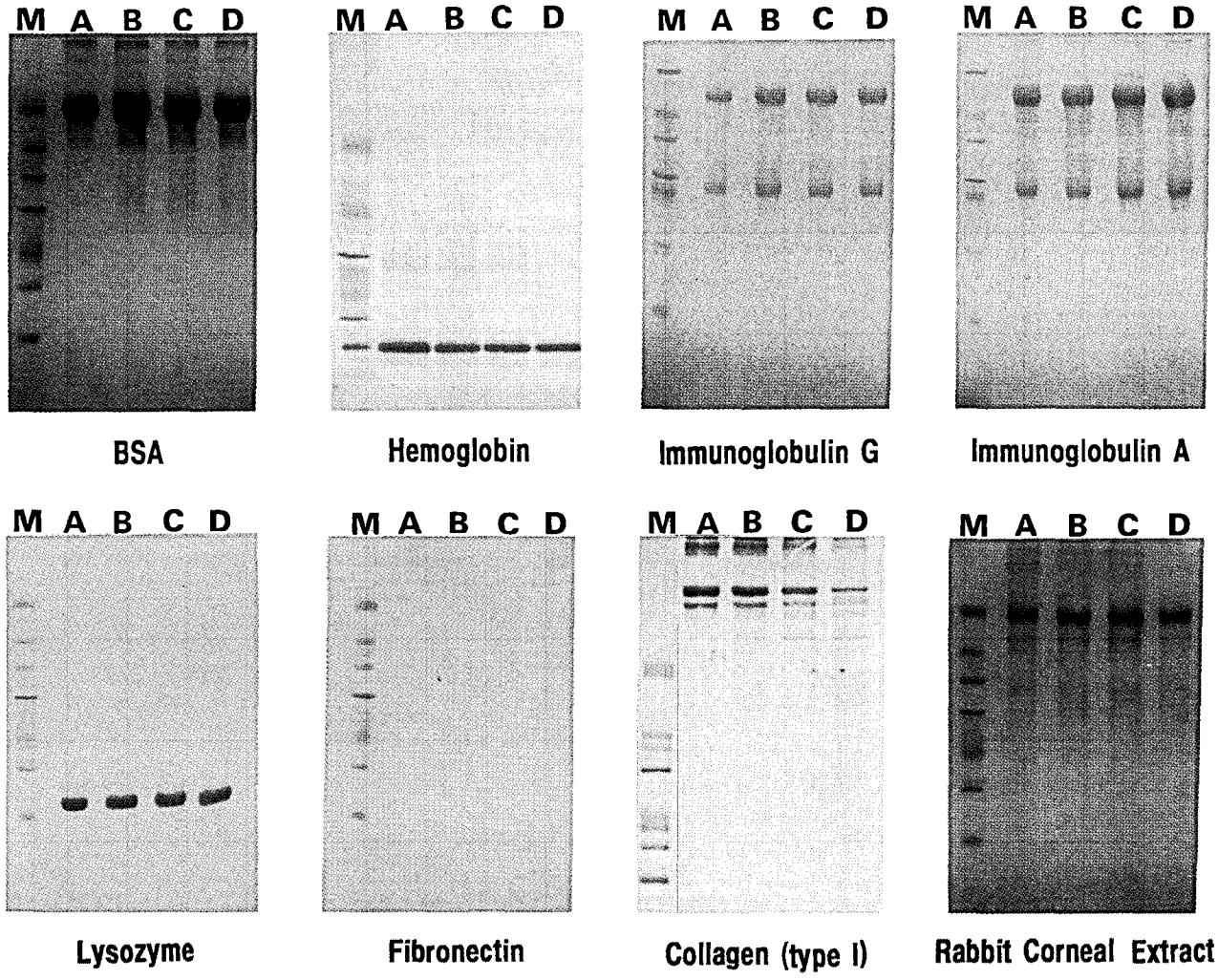

Fig. 6. Substrate specificity of the purified enzyme. The purified enzyme (10 μg) was increased with various substrate solutions (90 μg, 1 mg/ml) and 20 μl aliquots withdrawn at times from 2 h to 8 h for analysis by SDS-PAGE. lane A, reaction mixture without enzyme; lanes B-D, incubated for 2, 4 and 8 h, respectively; lane M, standard marker proteins.

ase (Muñoz *et al.*, 1982). Several investigators had hypothesized that invasion and tissue destruction by virulent strains of *Entamoeba* required expression and release of a histolytic proteinase (Neal, 1960; McLaughlin and Faubert, 1977). Keene *et al.* (1990) confirmed that the purified proteinase produced a cytopathic effect and specific, irreversible inhibitor of cysteine proteinase could prevent destruction of tissue culture monolayer. Proteinase activity has been reported in various species of *Acanthamoeba*. Jarumilinta and Maegraith (1961) described caseinase and gelatinase activities at pH 7.6 and Auriault and Desmazeaud (1979) described acid proteinase in extracts of *A. culbertsoni* and *A. rhysodes* which hydrolyze hemoglobin at pH 3.8.

In this study, we purified a serine proteinase from *A. culbertsoni* with activity which highly inhibited by serine specific inhibitors, DFP and PMSF. It was a neutral proteinase with an optimal pH of 7.0. Optimal temperature was 37℃. The molecular weight of the native enzyme estimated to be 108.0 kDa by Sephacryl S-200 HR gel filtration chromatography.

The amoeba cysteine proteinase had a broad substrate specificity, with activity against casein, gelatin, insulin, and of more biologically important type I collagen, fibronectin, and lamin (Keene *et al.*, 1986; Schulte *et al.*, 1987; Luaces and Barrett, 1988; Scholze and Schulte, 1988). The serine proteinase purified by us had a narrow substrate specificity against type I collagen and fibronectin.

Collagens are a superfamily of extracellular matrix proteins with a structural role as their primary function. Particularly, type I collagen is abundantly found in dermis, tendon, bone, and blood vessel walls and normally functions as a structural protein to maintain tissue integrity (Miller and Gay, 1982). Therefore, degradation of collagens by the enzyme may lead to tissue damage.

This suggested the possibility that tissue injury caused by acanthamoebal infection may due to degradation of collagens by this enzyme. Fibronectins are high molecular weight glycoproteins found in many extracellular matrices and in blood plasma. Therefore, degradation of fibronectin by the enzyme also related to tissue injury observed with acanthamoebal infection. Similar conclusions were drawn about the proteinases of *A. castellanii* and *A. polyphaga*. They had a collagenolytic activity and seemed to be related to destruction of host-protective barriers and tissue invasion of the organisms (He *et al.*, 1990; Mitro *et al.*, 1994). Therefore, proteinase purified from *A. culbertsoni* could be play a role as a virulence factor which damages host tissue and promotes amoeba tissue invasion and destruction by damaging tissue constituent proteins. This hypothesis was partially supported by observing degradation of rabbit corneal extract in this study.

However, to confirm the hypothesis more clearly, several problems must be solved. First, the proteinases thought to be highly involved in tissue invasion and destruction are extracellular proteinases rather than intracellular proteinases. One possible explanation for it is obtained from elastase of *Acanthamoeba* spp.. Elastase of pathogenic *Acanthamoeba* spp. could participate in destruction of host-protective barriers and promote amoeba tissue invasion and destruction (Ferrante and Bates, 1988). *Acanthamoeba* has a lot of granules containing various enzymes, including elastase. These granules exocytosed after cellular surface membrane stimulation. This may occur when amoeba attach to host tissues and the capacity to release enzyme could be a better reflection of amoeba pathogenicity. One possibility that the proteinase is stored in such granules in the same way of elastase and release to environments. However, to test this possibility, the biosynthetic pathway, cellular distribution, and release mechanism of the proteinase must be elucidated. Second, more detailed studies, including *in vivo* studies with inhibitors in order to test the hypothesis that this acanthamoebal proteinase is directly responsible for the acanthamoebal pathology, must be accomplished to confirm the role of proteinase as a virulence factor.

References

Aldape, K., Huizinga, H., Bouvier, J. and McKerrow, J. (1994) *Exp. Parasitol.* **78**, 230.

Auriault, C. and Desmazeaud, M. (1979) *Protistologica* **15**, 123.

Brooks, S. E. and Schneider, D. L. (1985) *J. Protozool.* **32**, 330.

Dunnebacke, T. H. and Trowan-Kelly, B. (1985) *J. Cell. Sci.* **75**, 1.

Ferrante, A. and Thong, T. H. (1983) *Clin. Exp. Immunol.* **54**, 477.

Ferrante, A. and Bates, E. J. (1988) *Infect. Immun.* **56**, 3320.

Gitler, C., Calef, E. and Rosenberg, I. (1984) *Philos. Trans. R. Soc. London* **B307**, 73.

He, Y.-G., Niederkorn, J. Y., McCulley, J. P., Stewart, G. L., Meyer, D. R., Silvant, R. and Daughtery, J. (1990) *Invest. Ophthalmol. Vis. Sci.* **31**, 2235.

Jarumilinta, R. and Maegraith, B. G. (1961) *Ann. Trop. Med. Parasitol.* **55**, 518.

Jones, D. B. (1986) *Am. J. Ophthalmol.* **102**, 527.

Keene, W. E., Petitt, M. G., Susan, A. and McKerrow, J. H. (1986) *J. Exp. Med.* **163**, 536.

Keene, W. E., Hidalgo, M. E., Orozco, E. and McKerrow, J. H. (1990) *Exp. Parasitol.* **71**, 199.

Laemmli, D. K. (1970) *Nature* **277**, 680.

Lawande, R. V., Abraham, S. N., John, I. and Egler, L. J. (1979) *Am. J. Clin. Pathol.* **71**, 203.

Lowry, O. H., Roseburge, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265.

Luaces, A. and Barrett, A. J. (1988) *Biochem. J.* **250**, 903.

Lushbauch, W. B., Hofbauer, A. F. and Pittman, F. E. (1984) *Gastroenterology.* **87**, 17.

Ma, P., Visvesvara, G. S., Martinez, A. J., Theodore, F. H., Dagett, P. M. and Sawyer, T. K. (1990) *Rev. Infect. Dis.* **12**, 490.

Martinez, A. J. (1985) Free Living Amoebas: Natural History, Prevention, Diagnosis, Pathology, and Treatment of Disease, CRC Press, Inc., Boca Raton, Florida.

McKerrow, J. H. (1987) *UCLA Sym. Mol. Cell. Biol. New Ser.* **42**, 553.

McKerrow, J. H., Sun, E., Rosenthal, P. and Bouvier, J. (1993) *Annu. Rev. Microbiol.* **47**, 821.

McLaughlin, J. and Faubert, G. (1977) *Can. J. Microbiol.* **23**, 420.

Miller, E. J. and Gay, S. (1982) *Methods Enzymol.* **82**, 2.

Mitro, K., Bhagavathiammai, A., Zhou, O. M., Bobbet, G., McKerrow, J. H., Chokshi, R. and James, E. R. (1994) *Exp. Parasitol.* **78**, 377.

Montfort, R. P., Saloma, P. O., Medina, L. V., Montfort, I. and Becker, I. (1987) *Mol. Biochem. Parasitol.* **26**, 87.

Muñoz, M. L., Calderon, J. and Rojkind, M. (1982) *J. Exp. Med.* **155**, 42.

Neal, R. A. (1960) *Parasitol.* **50**, 531.

Scholze, H. and Schulte, W. (1988) *Biomed. Biochim. Acta.* **47**, 115.

Scholze, H. and Schulte, W. (1989) *J. Protozool.* **36**, 538.

Schulte, W. Scholze, H. and Werries, E. (1987) *Mol. Biochem. Parasitol.* **25**, 39.

Theodore, F. H., Jakobiec, F. A., Juechter, K. B., Ma, P., Yroutman, R. C., Pang, P. M. and Iwamoto, T. (1985) *Ophthalmol.* **2**, 1471.

Willaert, E. and Leray, D. (1973) *Protistologica.* **9**, 417.