Journal of Biochemistry and Molecular Biology, Vol. 31, No. 3, May 1998, pp. 303–306

JOURNAL OF
Biochemistry and Molecular Biology

Cloning and Expression of a Serine Proteinase Gene Fragment from *Acanthamoeba culbertsoni*

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Received 16 February 1998

Serine proteinase cDNA fragment from protozoan parasite *Acanthamoeba culbertsoni* was amplified by the reverse transcription-polymerase chain reaction (RT-PCR) using degenerate oligonucleotide primers derived from conserved serine proteinase sequences. The amplified DNA fragment was subcloned and sequenced. The sequence analysis and alignment showed significant sequence similarity to other eukaryotic serine proteinases and conservation of the His, Asp, and Ser residues that form the catalytic triad. The cDNA fragment was cloned into the pGEMEX-1 expression vector and expressed in *Escherichia coli*. A resulting fusion protein of 56 kDa had proteolytic activity. The fusion protein reacted with sera of mice immunized with purified serine proteinase of *A. culbertsoni* in Western blot. Immune recognition of the fusion protein by mouse antisera suggested that the fusion protein may be valuable as a diagnostic reagent.

Keywords: *Acanthamoeba culbertsoni*, Cloning, Expression, Serine proteinase.

Introduction

Acanthamoeba are normally inocuous, free-living aquatic protozoa. However, given access, many species colonize human tissue and cause diseases such as chronic granulomatous amoebic encephalitis (GAE), acanthamoebic keratitis, pneumonitis, and subacute granulomatous dermatitis (Lawande *et al.*, 1979; Ma *et al.*, 1990; Martinez, 1991). During acanthamoebal infection in the various body sites, there is evidence of extensive tissue destruction. This may be due to cytopathogenic substances, oxygen radicals, and a number of different enzymes produced by *Acanthamoeba* (Bond and Butler, 1987; Ferrante and Bates, 1988).

Parasite proteinases have been known to be important virulence factors in the pathogenicity of many parasitic infections either by inducing tissue damage and facilitating invasion or by empowering the parasite to salvage metabolisms from host proteins (McKerrow, 1989). Different types of proteinases are frequently expressed at different stages of the parasite life cycle to support parasite replication and metamorphosis. Furthermore, parasite proteinases may be one of the critical factors driving the molecular evolution of host serum proteinase inhibitors.

In the case of *Acanthamoeba*, proteinases have previously been reported as being important virulence factors. Proteinases of *Acanthamoeba* trophozoites caused damage to collagen shields in an *in vitro* and *in vivo* assay in rat corneas (He *et al.*, 1990; Mitro *et al.*, 1994). Park and Song (1996) described that the purified proteinase from *A. culbertsoni* plays a role as a virulence factor which damages host tissue and promotes amoeba invasion by damaging tissue constituent proteins. Furthermore, secreted proteinases of *A. culbertsoni* exhibited strong cytopathogenicities to cultured animal cells (unpublished data).

In this study, we cloned a serine proteinase gene from *A. culbertsoni*. The partial gene was expressed in *E. coli*. Isolation and expression of this gene would be useful to study the antigenicity of serine proteinase of *A. culbertsoni* and the analysis of its structure, function, and molecular evolution.

Materials and Methods

Culture and harvest *Acanthamoeba culbertsoni* was kindly provided by the Department of Parasitology, College of Medicine, Yonsei University, and cultured in CGV medium at

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37°C for 7–8 d (Willaert and Leray, 1973; Park and Song, 1996). The cells were harvested by centrifugation at 3000 rpm for 30 min and washed several times with physiological saline solution.

Preparation of mRNA mRNA was prepared from *A. culbertsoni* cells using the Quick Prep™ Micro mRNA Purification Kit (Pharmacia, Uppsala, Sweden) following the manufacturer's instruction.

cDNA synthesis and polymerase chain reaction The synthesis of single-stranded cDNA from isolated mRNA was carried out using the cDNA Synthesis Kit (Boehringer Mannheim GmbH, Germany). Double-stranded cDNA fragment was amplified by PCR from single-stranded cDNA using two degenerate primers designed upon the consensus sequence of amino acids flanking the active site serine and histidine residues of eukaryotic serine proteinase (Sakanari *et al.*, 1989). The forward primer was 5′-ACAGAATTCTGGGTIGTIACIGCIGCICAYTG-3′ and the reverse primer was 5′-ACAAAGCTTAXIGGICCICCI(CX, GA)XTCICC-3′, where X is A or G, Y is T or C, and I is inosine. Restriction sites *Eco*RI and *Hind*III were added to the 5′-ends of each primer to allow cloning in a known orientation and subsequent sequencing. Amplification reactions were performed by 32 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min, and the final extension step was 10 min at 72°C in a DNA Thermal Cycler (Model 480, Perkin-Elmer Cetus, USA). The amplified fragment was purified from the gel with a Geneclean Kit (Bio 101, Vista, USA) and directly cloned in an Original TA cloning vector (Invitrogen, Sandiego, USA).

DNA sequencing and sequence analysis The nucleotide sequence of the plasmid insert was determined by the dideoxy nucleotide chain termination method (Sanger *et al.*, 1977), using a Sequenase version 2.0 DNA Sequencing Kit (United States Biochemicals, USA). Nucleotide and amino acid sequences analyses were carried out using the DNASIS and GenEMBL database (Pharmacia).

Expression of fusion protein The partial cDNA fragment obtained by PCR was purified, digested with *Eco*RI and *Hind*III, and ligated into pGEMEX-1, which had been digested with *Eco*RI and *Hind*III. The resulting plasmid was transformed into *E. coli* JM109 competent cells (Pharmacia) by standard methods and spread on plates containing 50 μg/ml of ampicillin (Sambrook *et al.*, 1989). A transformed cell was selected and confirmed for the existence of insert by sequencing. Expression of fusion protein was induced by isopropyl-β-D-thiogalactoside (IPTG) and analyzed by SDS-PAGE (Laemmli, 1970).

Assay of enzyme activity of fusion protein Proteinase activity was estimated by using synthetic dipeptide substrate with a fluorescent leaving group, carboxybenzoyl-arginine-arginine-7-amino-4-trifluoromethyl-coumarin (CBZ-arg-arg-AFC, Enzyme System Products, USA) as previously described (Park and Song, 1996).

Substrate gel electrophoresis The slab gel containing SDS and gelatin as copolymerized substrate was used for the detection of the proteinase activity on the gel (Montfort *et al.*, 1987). Protein samples were mixed with an equal volume of sample buffer containing 2.5% SDS, 1% sucrose, and 0.05% bromophenol blue followed by incubation at 37°C for 30 min. After electrophoresis, the gel was soaked in 2.5% Triton X-100 for 1 h at room temperature to remove SDS and restore enzyme activity. The gel was then transferred to 0.1 M Tris-HCl buffer (pH 7.0) and incubated at 37°C overnight. The gel was stained with Coomassie blue and destained. The band of proteolytic activity was revealed as the depleted area of gelatin.

Preparation of mouse antisera Mouse antisera were prepared by immunizing female BALB/c mice with the following method. One hundred microliters of purified *A. culbertsoni* serine proteinase antigen in sterile physiological saline (100 μg/ml) was mixed with an equal volume of Freund's complete adjuvant (Difco Laboratories, Detroit, USA) and injected intraperitoneally into BALB/c mice. The second and third injections were performed under the same condition except that Freund's incomplete adjuvant (Difco) was used after 2 and 4 weeks after the first injection. Three days prior to sacrifice, mice were boostered with 100 μl of antigen by intravenous injection into the tail vein.

Western blot After SDS-PAGE, the fusion protein was transferred onto nitrocellulose membrane (Tsang *et al.*, 1983) and then blocked with 3% skim milk in PBS-Tween 20 (PBST, 0.05% Tween 20 in phosphate buffered saline, pH 7.2). After washing twice with PBST, the membrane was incubated in 1:100 diluted antiserum at room temperature for 2 h and washed with PBST three times. The membrane was incubated with peroxidase-conjugated anti-mouse IgG diluted in 1:1000 with PBST. The reaction was visualized by incubating the membrane with substrate solution of 0.05% 3,3′-diaminobenzidine (Sigma, USA) and 0.01% hydrogen peroxide. The reaction was stopped by the addition of distilled water.

Results and Discussion

A partial gene fragment encoding serine proteinase of *A. culbertsoni* was cloned and expressed in *E. coli*. PCR was carried out for isolation of the serine proteinase gene fragment from *A. culbertsoni* using the degenerate primers designed on the basis of the consensus sequence of amino acids flanking the active site serine and histidine residues of eukaryotic serine proteinase (Sakanari *et al.*, 1989). The amplified product was ligated into the TA cloning vector and sequenced. The nucleotide sequence and deduced amino acid sequence are shown in Fig. 1. In the deduced amino acid sequence of the serine proteinase cDNA fragment, serine, aspartic acid, and histidine residues, which form the catalytic triad of serine proteinase, are conserved. High homology was observed around the serine and histidine residues when compared to other reported serine proteinases such as those of *Schistosoma mansoni* cercaria (Newport *et al.*, 1988), *Tripanosoma cruzi* (Sakanari *et al.*, 1989), venome plasminogen activator (Zhang *et al.*, 1995), and various human and mammalian-

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1    ACA GAA TTC TGG GTG GTG ACG GCG GCC CAC TGC TGG GCT ACC ACA TAT   48
1      EcoRI      W  V  V  T  A  A  H  C  W  A  T  R  Y               16

49   CAA TCT TCT TCT GGT GTC TGT AAT TGG AGG TTC GAT CTT CAA GGT TCT   96
17   Q  S  S  S  G  V  C  N  W  R  F  D  L  Q  G  S                  32

97   CAA GAT CAT TCT GTC AAA ACC GCC GGA CTT AAT AGA CCT TCT GGC CTC  144
33   Q  D  H  S  V  K  T  A  G  L  N  R  P  S  G  L                  48

145  ATC CCT GCC TCA GCA ATC AGT CTT TTT CAT CAA TTA CAT GCT TGT GGT  192
49   I  P  A  S  A  I  S  L  F  H  Q  L  H  A  C  G                  64

193  TGG CCT AGG TCG TGG GCG CTC AAA CTC ATG CGC TAC ATG GCA TTT GTA  240
65   W  P  R  S  W  A  L  K  L  M  R  Y  M  A  F  V                  80

241  AAG CTT TAC TCG CGT CTT TTG CTC TCA TGG CTC AGG GGT AGA ACT GCG  288
81   K  L  Y  S  R  L  L  L  S  W  L  R  G  R  T  A                  96

289  GAG GAG CGA CAG GAG ATA AGA CAG CCT AGA CAG TTC GAT TAT GCC GAT  336
97   E  E  R  Q  E  I  R  Q  P  R  Q  F  D  Y  A  D                  112

337  CAC GTG TCA ATG GAT GTG CTG ATC TTC TCA GTG GTG CTC ATC TTT AGT  384
113  H  V  S  M  D  V  L  I  F  S  V  V  L  I  F  S                  128

385  GTT ATG GCA CCT CGA TCG CCT GCT TTG GCA TTA TCT ACT TCT TCT TTG  432
129  V  M  A  P  R  S  P  A  L  A  L  S  T  S  S  L                  144

433  CTT TTC TGG CTC ACC GGT ACA ACA TCA TCC ATG TCT ATC GTG CGC GTT  480
145  L  F  W  L  T  G  T  T  S  S  M  S  I  V  R  V                  160

481  ACG AGT CCG GCG GCA TCA TGT GGC ACT CGA TAT TCA TTT CAT TAT GGC  528
161  T  S  P  A  A  S  C  G  T  R  Y  S  F  H  Y  G                  176

529  TGC TCT TAT GCT CTT CCA GCT CCC ATG GCA GGT GTA CTG GGA GCG AAG  576
177  C  S  Y  A  L  P  A  P  M  A  G  V  L  G  A  K                  192

577  GGC TAT GGC GAC TCC GGC GGC CCC CTA AGC TTT GT                   608
193  G  Y  G  D  S  G  G  P      Hind III                            202
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Fig. 1. Nucleotide sequence of *A. culbertsoni* serine proteinase cDNA fragment and its deduced amino acid sequence. The degenerated primer sequences are underlined. The serine proteinase catalytic triad of residues of H, D, and S are shown in the bold face type.

derived serine proteinases (Gershenfeld and Weissman, 1986). However, the sequences around aspartic acid showed low homology to each other (Fig. 2). Unfortunately, we failed to obtain full-length cDNA of serine proteinase; however, a partial serine proteinase gene fragment obtained by this study provided a probe to obtain the full-length cDNA and gene.

For further study, the cloned gene was expressed in *E. coli*. The cloned gene fragment was digested with *Eco*RI and *Hind*III, ligated to the pGEMEX-1 which had been digested with the same restriction enzymes, and transformed into *E. coli* JM109 competent cells. The expression of the target gene was induced by addition of

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A. culbertsoni      -- VTAAHCWAT --- GSQDHSVKTA --- AKGYGDSGGP --
S. mansoni          -- LTAGHCVCS --- LSGDIAIVML --- LPAPGDSGGP --
T. cruzi            -- VTAAHCWRI --- GISDAQWAKG --- DIVIGDSGGP --
Anisakis            -- VTAAHCYKS --- INNDIMLIQL --- DSCQGDSGGP --
Human tPA           -- LSAAHCFQE --- YDNDIALLQL --- DACQGDSGGP --
Human plasminogen   -- LTAAHCLEK --- TRKDIALLKL --- DSCQGDSGGP --
Human Factor IX     -- VTAAHCVET --- YNHDIALLEL --- DSCQGDSGGP --
Venom PA            -- VTAAHCDSN --- DEVDKDIML1 --- TCHFGDSGGP --
Mouse H Factor      -- LTAAHCNVG --- REGDLQLVRL --- DSCNGDSGGP --
Rat elastase I      -- MTTAHCVSS --- AGYDTALLRL --- SSCNGDSGGP --
Rat trypsin II      -- VSAAHCYKS --- LNNDIMLIKL --- DSCQGDSGGP --
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Fig. 2. Comparison of amino acid sequences surrounding the active site catalytic triad of serine proteinase of *A. culbertsoni* and other serine proteinases. Alignment of these fragments shows that they contain the conserved residues of the catalytic triad of serine proteinases (shown in bold type). PA, plasminogen activator; tPA, tissue plasminogen activator.

IPTG and analyzed on SDS-PAGE followed by Coomassie blue staining. The size of the expressed protein was approximately 56 kDa, corresponding to the predicted size (Fig. 3). To investigate whether the expressed protein has enzyme activity, the proteolytic enzyme activity was estimated. Interestingly, the expressed protein had proteolytic enzyme activity even though it had only core region constructing active sites (Table 1). The enzyme activity was highly inhibited by PMSF, a serine proteinase inhibitor. The fact that the fusion protein had proteolytic activity was also confirmed in gelatin SDS-PAGE analysis (Fig. 4). These suggested that the fusion protein has a characteristic of serine proteinase although many parts of the enzyme were deleted. The fusion protein reacted with sera of mouse immunized with purified serine proteinase of *A. culbertsoni*, but not with normal sera (Fig. 5). Therefore, the fusion protein was thought to be a true product of the cloned serine proteinase gene fragment. Immune recognition of the fusion protein by mouse antisera suggests that the fusion protein may be valuable as a diagnostic reagent.

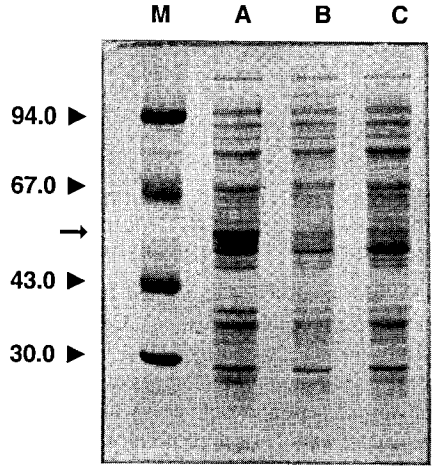

Fig. 3. SDS-PAGE analysis of the expressed serine proteinase. Lane M, molecular weight standard proteins; Lane A, induced cell extract; Lane B, uninduced cell extract; Lane C, host cell extract. An arrow to the left of the gel indicates the recombinant fusion protein of serine proteinase.

Table 1. Proteinase activity of the expressed *A. culbertsoni* serine proteinase.

Strain	Specific activity (U/mg)[a]	
	−PMSF	+PMSF
Host strain JM109	3.3 ± 0.7	0.7 ± 0.2
Transformed JM109 (uninduced)	4.0 ± 0.9	0.8 ± 0.1
Transformed JM109 (induced)	16.9 ± 1.5	0.8 ± 0.2

The cell extracts were prepared by sonication in PBS (pH 7.2) on ice.

[a] nmoles of AFC production per min under reaction conditions.

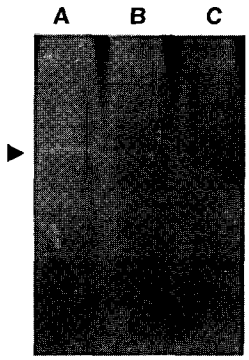

Fig. 4. Substrate gel electrophoresis analysis of the expressed serine proteinase. Lane A, induced cell extract; Lane B, uninduced cell extract; Lane C, host cell extract. An arrow to the left of the gel indicates the recombinant fusion protein of serine proteinase.

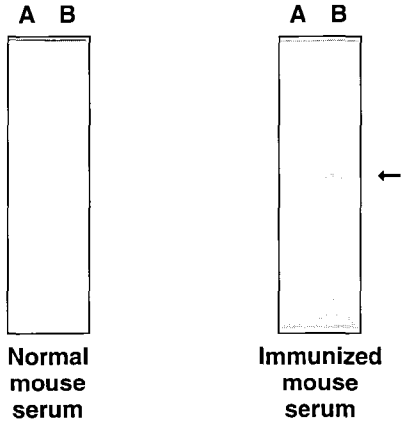

Fig. 5. Western blot analysis of expressed fusion protein. Lane A, Cell lysate of uninduced cells; Lane B, Cell lysate of induced cells. Cell lysates were separated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were incubated with antiserum and normal serum. A bold arrow to the right of the gel indicates the expressed fusion protein which reacted with antiserum.

We plan to clone the full gene encoding serine proteinase of *A. culbertsoni* by reconstructing its cDNA library and expressing the gene. Characterization of the overexpressed gene product will provide biochemical information on the function of the enzyme and the role it may play in the pathogenesis of *Acanthamoeba*.

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