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ORIGINAL RESEARCH PAPER

Screening and characterization of a novel fibrinolytic metalloprotease from a metagenomic library

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Abstract A metagenomic library was constructed using total genomic DNA extracted from the mud in the west coast of Korea and was used together with a fosmid vector, pCC1FOS in order to uncover novel gene sources. One clone from approximately 30,000 recombinant *Escherichia coli* clones was identified that showed proteolytic activity. The gene for the proteolytic enzyme was subcloned into pUC19 and sequenced, and a database search for homologies revealed it to be a zinc-dependent metalloprotease. The cloned gene included the intact coding gene for a novel metalloproteinase and its own promoter. It comprised an open reading frame of 1,080 base pairs, which encodes a protein of 39,490 Da consisting of 359 amino acid residues. A His-Glu-X-X-His sequence, which is a conserved sequence in the active site of zinc-dependent metalloproteases, was found in the deduced amino acid sequence of the gene, suggesting that the enzyme is a zinc-dependent metalloprotease. The purified enzyme showed optimal activity at 50°C for 1 h and pH 7.0. The enzyme activity was inhibited by metal-chelating reagents, such as EDTA, EGTA and 1,10-phenanthroline. The enzyme hydrolyzed azocasein as well as fibrin. Thus, the enzyme could be useful as a therapeutic agent to treat thrombosis.

Keywords Azocasein · Fibrinolytic · Metagenome · Metalloprotease

The sequence reported in this paper has been deposited in the GenBank database (Accession number: EF100137).

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Introduction

Proteases are ubiquitous enzymes (Sharma et al. 2004) and are of major importance in the food, leather, detergent, pharmaceutical and waste management industries and for diagnosis of illness. Proteases constitute two thirds of the total number of enzymes used in industry and this is expected to increase (Gupta et al. 2002). In addition, a number of proteases that can interfere with blood homeostasis have been purified and characterized from various sources. Some of these proteases are fibrinolytic enzymes capable of digesting fibrin (Fujita et al. 1993; Jeong et al.

2004; Leonardi et al. 2002; Sumi et al. 1995; Wong and Mine 2004). The fibrinolytic agents available today for clinical use are mostly plasminogen activators (Holden 1990), such as tissue-type plasminogen activator, urokinase-type plasminogen activator and the bacterial plasminogen activator, streptokinase. In spite of their widespread use, these agents display low specificity to fibrin and cause undesired side effects. Consequently, the search continues for other fibrinolytic enzymes from various sources for use in thrombolytic therapy.

Screenings for novel enzymes, including proteases, have mainly used the cultivation-dependent approach. Many valuable enzymes originated from cultivable microorganisms; however, the rate of screening for novel enzymes is significantly decreased when standard cultivation methods are used owing to a high rediscovery frequency (Strohl 2000). The structure of microbial communities in various environments is diverse and complex. Nonetheless, the cultivable microorganisms constituting these resources correspond to only a small fraction of the microbial diversity—less than 1% of the microorganisms in various environments are readily cultivable (Amann et al. 1995). This limits the range of a search for new biocatalysts for the bioprocess industry, so the use of complex communities and the effort to overcome the problem of non-cultivability attract not only scientific attention but also biotechnological innovation.

Metagenomes are the genomes of non-cultivatable microorganisms existing within a certain environmental microbial community. Hence, the generation and analysis of metagenomic libraries is a powerful approach to the collection and archiving of environmental genetic resources (Ferrer et al. 2005). Methods have been developed and used to overcome the non-cultivability of environmental microorganisms for biotechnology, namely cloning and the expression of metagenomes in suitable expression hosts.

In order to discover new proteases from metagenomic libraries, we screened for proteolytic activity from a constructed metagenomic library by direct cloning of environmental DNA of large DNA inserts. A novel gene encoding a zinc-dependent proteolytic enzyme was picked up, sequenced, expressed in *E. coli* and characterized.

Materials and methods

Bacterial strains and plasmids

Escherichia coli EPI300 (Epicentre, Madison, WI, USA) was used as a host, with a fosmid, pCC1FOS (Epicentre) as a vector in order to construct the deep-sea sediment library. pUC19 was used for subcloning and construction of the expression plasmid. *E. coli* DH5α (*supE*44, Δ*lac*U169(*ϕ*80 *lacZ*ΔM15), *hsdR*17, *recA*1, *EndA*1, *gyrA*96, *thi*-1, *relA*1) was used as the host for manipulations and expression of the gene. *E. coli* cells were routinely grown at 37°C in Luria–Bertani (LB) broth (Difco), supplemented with 100 μg ampicillin/ml when required.

Metagenomic library construction and screening of the protease gene

Deep-sea sediment samples from a clam bed community were obtained as described by Lee et al. (2004). The DNA extraction method of Hurt et al. (2001) was used for DNA isolation from deep-sea sediment samples with minor modifications, and further purification of the DNA was performed by direct extraction from the agarose gel. The construction of the metagenomic library was performed according to the manufacturer's protocol. Sheared and end-repaired DNA was ligated into pCC1FOS (Epicentre), and the ligated DNA was packaged using MaxPlax Lambda Packaging Extracts (Epicentre). *E. coli* EPI300 (Epicentre) cells were infected using packaged DNA and plated on LB agar medium supplemented with 12.5 μg chloramphenicol/ml and 1% (w/v) skim milk for the direct screening of protease activity.

Subcloning and DNA sequencing of the protease gene

The methods used for molecular cloning were based on those of Sambrook et al. (1989). Fosmid DNA was isolated from positive colonies and

digested with *Eco*RI and *Sph*I, and DNA fragments of 2–5 kb were ligated into the corresponding sites of pUC19. The ligated DNA was transformed into *E. coli* DH5α and the transformants were examined for proteolytic activity using LB agar medium supplemented with 100 μg ampicillin/ml and 1% (w/v) skim milk. Plasmid DNA (termed pUC-ES63H9) was isolated from a proteolytic clone and sequenced at Bionex Inc. (Seoul, Korea). Sequence analysis was carried out using the DS Gene 1.5 program (Accelrys Inc., San Diego, CA, USA).

Construction of an *E. coli* expression vector using the promoter region of the clone

pUC-ES63H9 was digested with *Eco*RI and *Bam*HI, and a 0.9 kb DNA fragment was ligated into the corresponding sites of pUC19, resulting in pUC-ES63H9pro. pTXB3 (New England Biolabs Inc., Beverly, MA, USA) was digested with *Nco*I and *Bam*HI, and a 0.8 kb DNA fragment was ligated into the corresponding sites of pUC-ES63H9pro, resulting in an *E. coli* expression vector, pES63H9pro3-MIC.

Expression and purification of recombinant protease

The putative protease gene was amplified from the pUC-ES63H9 plasmid with Pyrobest DNA polymerase (Takara Bio Inc., Otsu, Japan). The primers used were ES63H9_E2-F (5′-GAATTC-CATGGAACCAGAACCGATC-3′) incorporating *Eco*RI and *Nco*I restriction sites (underlined) at the 5′-end and ES63H9_E1-R (5′-GCGGCCG-CGCTCCGCCGCGTCATCCCTATAG-3′) with a *Not*I restriction site (underlined) at the 5′-end. Amplified DNA was ligated to pGEM-T Easy vector (Promega, Madison, WI, USA), resulting in pGEMTe-ES63H9_E21. pGEMTe-ES63H9_E21 carrying the protease gene was digested with *Nco*I and *Not*I, and a 1.1 kb DNA fragment was ligated to the corresponding sites of the constructed expression vector, pES63H9pro3-MIC. The recombinant plasmid was introduced into *E. coli* DH5α cells, which were then grown overnight, collected by centrifugation at 5,000 × *g* for 5 min, and used for plasmid preparation. The integrity of the recombinant plasmid was confirmed by restriction digestion using *Nco*I and *Not*I and designated pES63H9pro3-ES63H9-MIC. *E. coli* DH5α cells harbouring pES63H9-pro3-ES63H9-MIC were grown in 1 l LB broth supplemented with 100 μg ampicillin/ml at 37°C for 12 h. The cells were collected by centrifugation at 5,000 × *g* for 5 min, and suspended in 30 ml of ice-cold column buffer (20 mM Tris/HCl, pH 7.4, 0.5 M NaCl, 0.2% Triton X-100, 2 mM EDTA). After cell disruption by sonication, the sample was centrifuged at 20,000 × *g* for 20 min, and the supernatant was purified as described in Table 1.

Enzyme assay

Protease activity was determined by measuring the release of acid-soluble material from azocasein (Sigma) (Windle and Kelleher 1997). All assays were conducted in 50 mM Tris/HCl, pH 7.0, buffer. Enzyme samples (100 μl) were added to 100 μl 1% (w/v) azocasein. The reaction mixture was incubated at 50°C for 1 h and terminated by the addition of 400 μl 10% (w/v) trichloroacetic acid. The precipitated protein was removed by centrifugation (12,000 × *g*, 5 min),

Table 1 Purification of metalloprotease from *E.coli* cells harbouring pES63H9pro3-ES63H9-MIC

Purification step	Total protein (mg/l)	Total activity (U)	Specific activity (U/mg)	Yield (%)
Cell-free extract	138	76,000	551	100
Affinity chromatography	1	3,400	3,400	4

The cell-free extract was put on a chitin bead column (20 ml of set volumes) (New England Biolabs Inc.) equilibrated with column buffer. The column was washed with the same buffer, and then equilibrated with a cleavage buffer (column buffer with 30 mM DTT) at 4°C overnight. Proteins were eluted with column buffer to a total volume of 50 ml. The amount of protein was measured using BCA protein assay reagent (Pierce Biotechnology, IL, USA), with bovine serum albumin as the standard protein

and the resulting supernatant was transferred to a clean tube containing 700 μl 525 mM NaOH. Absorbance was measured at 442 nm. One unit of protease activity was defined as amount required to produce enough acid-soluble material from azocasein to yield an absorbance of 0.1 at 442 nm, following 1 h incubation at 50°C.

Fibrinolytic assay

Fibrinolytic activity was determined using the method of Datta et al. (1995), with minor modifications: 10 μl 1% (w/v) human fibrinogen (Sigma) (prepared in 20 mM Tris/HCl, pH 7.4, buffer) was added to human thrombin (0.05 NIH unit, Sigma), and then allowed to stand for 1 h at 25°C. Clots which formed were mixed with purified enzyme and incubated at 37°C. At intervals, 5 μl samples were analyzed using an 11% (v/v) SDS-PAGE gel (see Fig. 4).

Results

Screening of the protease gene

A DNA library was constructed in a fosmid vector, pCC1FOS using a sediment sample collected from a deep-sea clam bed community. This library was screened for proteolytic activity of the clones on a skim milk agar plate. As a result, a protease-positive clone, pES63H9 was selected.

Subcloning and DNA sequences analysis of the protease gene

The protease gene from pES63H9 was subcloned into pUC19 and sequenced as described in Materials and methods. The gene is comprised of 1,080 bp with a G + C content of 54.5% (access no. EF100137 at NCBI). The gene begins with ATG and ends with TAA (Fig. 1). No typical Shine-Dalgarno or tandem inverted repeat sequences were found in the 5′- and 3′-noncoding regions, respectively. The gene encodes a protein of 359 amino acids with a molecular mass of 39,490 daltons (Fig. 1). The amino acid sequence was 46% identical to metallopeptidase from *Dechloromonas aromatica* (accession no. AAZ45577 at NCBI). By conserved domain searching (Marchler-Bauer et al. 2005), a His-Glu-X-X-His sequence (where X is any non-conserved amino acid) was found at positions 150–154 of the enzyme (Fig. 1). This is a conserved sequence in the active site of zinc-dependent metalloproteases. These findings suggest that the enzyme is a zinc-dependent metalloproteinase.

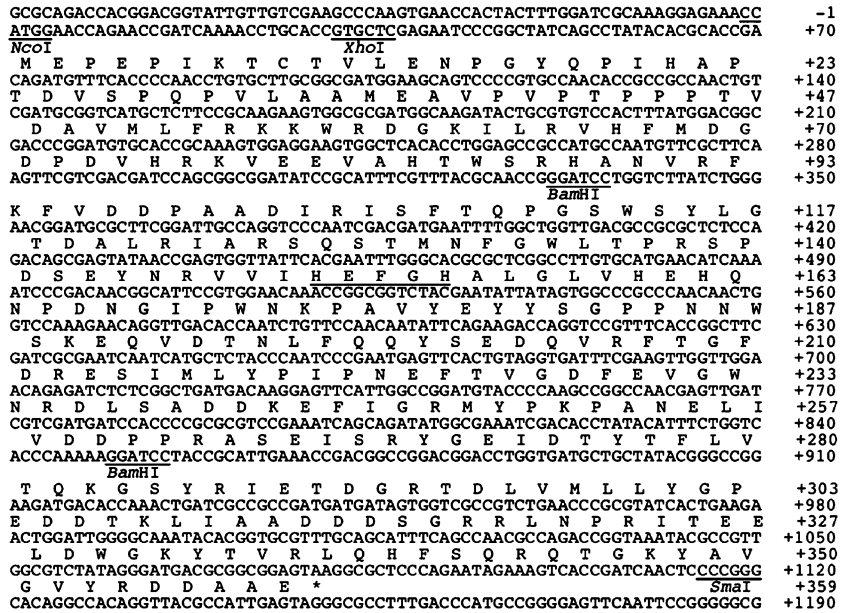

Fig. 1 Nucleotide sequence of the protease clone and deduced amino acid sequence of the enzyme. The nucleotide sequence of the protease gene and its flanking regions are shown (access no. EF100137 at NCBI). The deduced amino acid sequence of the gene product is indicated by the single-letter codes under the nucleotide sequence. A conserved sequence in the active site of zinc-dependent metalloproteases is underlined. Some unique restriction sites are shown

Overexpression and purification of recombinant ES63H9

Production of recombinant protease was examined using *E. coli* DH5α as a host and pES63H9pro3-MIC as a vector. *E. coli* DH5α cells harbouring pES63H9pro3-ES63H9-MIC produced a high amount of protease (Fig. 2). The recombinant protease was purified 6.3-fold after affinity chromatography, with a specific activity of 76,000 U/mg and a final yield of 4.4% (Table 1). The SDS-PAGE of the purified enzyme exhibited a single band with an apparent molecular mass of 39 kDa (Fig. 2). This value agreed with that estimated from the DNA sequence.

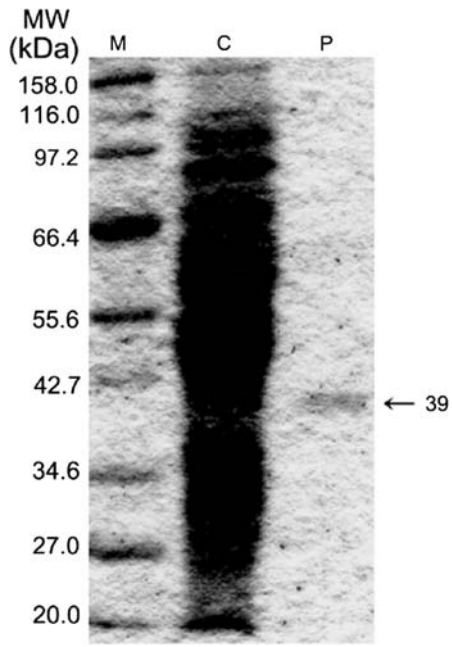

Fig. 2 SDS-PAGE of protease from *E. coli* cells harbouring pES63H9pro3-ES63H9-MIC. SDS-PAGE was performed by the Laemmli method with an 11% polyacrylamide gel. The enzyme solution was mixed with the sample buffer and boiled for 5 min before being placed on the gel. The gels were stained for protein with GelCode Blue Stain Reagent (Pierce, Rockford, IL, USA). Lane M, size marker, lane C, cell-free extract, lane P, purified enzyme (see Table 1). The arrow indicates the position of protease

Effects of temperature and pH on enzyme activity and stability

The optimal temperature for the activity of protease was 50°C for 1 h (Fig. 3A). Enzyme activity was 26% of maximum at 40°C and 18% at 60°C. The enzyme therefore is maximally active over narrow range of temperature. The optimal pH for the activity of protease was 7.0 (Fig. 3B).

Effects of metal ions and chemical reagents on enzyme activity

The effects of various metal ions on enzyme activity are shown in Table 2. Activities were enhanced by Co^{2+}, Ca^{2+} and Ni^{2+} ions, but inhibited by Mg^{2+} and Zn^{2+} ions. Activities were also inhibited by 1 mM EDTA, EGTA and 1,10-phenanthroline, well-known metalloprotease inhibitors. The enzyme was easily denatured by 0.05% SDS and strongly inhibited by 0.5 M guanidium hydrochloride. PMSF, a serine protease inhibitor, had no influence. These results all support the idea that the enzyme was a metalloenzyme.

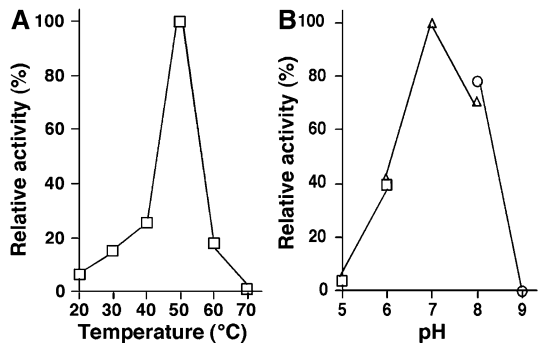

Fig. 3 Effects of temperature and pH on activity of recombinant protease. (**A**) Temperature dependence of the enzyme activity. The optimal temperature for protease activity was examined in the buffer used in the standard assay at various temperatures. The values on the ordinate are shown as percentages of the enzyme activity (100%, 3,400 U/mg) observed at 50°C for 1 h. (**B**) pH dependence of the enzyme activity. The optimal pH for protease activity was determined in various buffers at 50°C. The buffers used were sodium acetate buffers (open rectangles, pH 5.0–6.0), Tris HCl buffers (open triangles, pH 6.0–8.0) and glycine NaOH buffers (open circles, pH 8.0–9.0). The values on the ordinate are shown as percentages of the enzyme activity (100%, 3,400 U/mg) observed at pH 7.0

Table 2 Effect of metal ions and chemical reagents on enzyme activity

Metal ion or reagent	Concentration (mM)	Relative activity (%)
None		100
$CoCl_2$	1	280
$CaCl_2$	1	200
$NiSO_4$	1	140
$CuSO_4$	1	100
$FeSO_4$	1	100
$ZnCl_2$	1	80
$MgCl_2$	1	20
EDTA	1	27
EGTA	1	21
1,10-phenanthroline	1	55
SDS	17	9
Guanidine hydrochloride	500	22
PMSF	1	104

The effects of metal ions were investigated using $CoCl_2$, $CaCl_2$, $MgCl_2$, $NiSO_4$, $CuSO_4$, $ZnSO_4$, and $FeSO_4$. The effects of chemical reagents were also assessed using ethylenediaminetetraacetic acid (EDTA), ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 1,10-phenanthroline, sodium dodecyl sulfate (SDS), guanidine hydrochloride, phenylmethylsulfonyl fluoride (PMSF) (All these chemical reagents were purchased from Sigma). The purified enzymes were pre-incubated in both the absence and the presence of metal ions or chemical reagents in 50 mM Tris/HCl, pH 7.0, buffer at 25°C for 30 min. After 30 min of pre-incubation, residual protease activity was measured with 1% (w/v) azocasein under the standard conditions in the presence of the same metal ion or reagent. Absolute activity corresponding to 100% is 3,400 U/mg

Fibrinolytic activity of the enzyme

The hydrolysis of fibrin by the enzyme was analyzed by SDS-PAGE (Fig. 4). The purified enzyme completely hydrolyzed the α-chain and the γ–γ-chain after 1 h at 37°C. The enzyme also partially hydrolyzed the β-chain after 30 min; however, it was not completely hydrolyzed after 2 h (Fig. 4) and even after 24 h (data not shown).

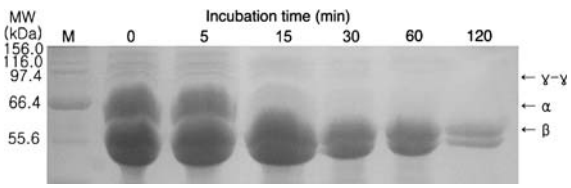

Fig. 4 Time-dependent hydrolysis of fibrin by recombinant protease. The enzyme solutions (17 U) were incubated with fibrin clots (20 mM Tris/HCl, pH 7.4, buffer) at 37°C for up to 120 min. The reaction mixtures were analyzed by SDS-PAGE gel

Discussion

In this work, a metagenomic library using a sediment sample collected from a deep-sea clam bed community was constructed in a fosmid vector pCC1FOS, and one novel protease gene was isolated. The one positive clone, pES63H9, possessed one complete gene, which encodes a putative protease. Although the gene was supposed to be expressed by its own promoter, no possible promoter sequence or Shine-Dalgarno sequence were found upstream of the gene. This is probably because the gene is from a microorganism that is phylogenetically distant from *E. coli*, and the ribosome-binding site may be quite different. The recombinant protease was produced using pES63H9pro3-MIC as a vector and *E. coli* DH5α as a host. When the protease gene was cloned with its 0.5-kb upstream region (Fig. 1), the protease was constitutively expressed in *E.coli* cells without the need for induction materials, such as IPTG (Ramos et al. 2003) or mitomycin C (Kim et al. 2004) (Fig. 2). This result indicates that the cloned gene contained its own promoter, which worked in *E.coli* cells. The recombinant protease had a molecular mass of 39 kDa and a specific activity of 3,483 U/mg. (Fig. 2, Table 1) Maximal activity of the enzyme was observed at 50°C and pH 7.0 (Fig. 3).

His-Glu-X-X-His, where X is any non-conserved amino acid, is the consensus sequence for the active-site in some zinc-dependent endopeptidases and aminopeptidases (Vallee and Auld 1990). The recombinant enzyme possessed a His-Glu-Phe-Gly-His sequence at amino acids 150–154, suggesting that it is a zinc-dependent metalloprotease (Fig. 1). Neutral zinc metallopeptidases (accession no. CDD16541 at NCBI) and astacin of crayfish (accession no. CDD24541), members of peptidase family M12A which require zinc for catalysis, were most similar to our enzyme. Bode et al. (1993) reported that astacins, metalloprotease, and snake venom

exhibited identical zinc-binding environments (His-Glu-X-X-His-X-X-Gly-X-X-His) and this was also a consensus sequence in metalloprotease disintegrins, another member of the zinc metalloprotease superfamily (Poindexter et al. 1999). In addition, His-Glu-X-X-His-Ala-Leu-Gly-X-X-His-Glu sequence is conserved among neutral zinc metallopeptidase family members (CDD16541) and this sequence was also found in our cloned protease (Fig. 1). The enzyme activities were inhibited by metal-chelating agents, such as EDTA, EGTA and 1,10-phenantroline (Table 2). The enzyme activities were enhanced by metal ions, such as Co^{2+}, Ca^{2+} and Ni^{2+}, but inhibited by Mg^{2+} and Zn^{2+} ions (Table 2). Lee et al. (1994) reported that the activity of zinc-dependent carboxypeptidase was activated by Co^{2+} ion, but inhibited by a high concentration (1 mM) of Zn^{2+} ion. There are other reports indicating that inhibition occurs if there is an excess of the corresponding ion, as additional binding of metal ions to the binding site lowers the activities of some metalloproteinases (Larsen and Auld 1991; Luciano et al. 1998).

Fibrinolytic enzymes have been purified from fermented food (Fujita et al. 1993; Jeong et al. 2004; Wong and Mine 2004), earthworms (Nakajima et al. 1993), and mushrooms (Kim and Kim 2001) as well as snake venom (Leonardi et al. 2002). These enzymes, which consist of both serine proteases and metalloproteases, have been suggested as potential sources of oral fibrinolytic drugs. Recently, fibrinolytic enzymes in shark cartilage extract have been characterized. These fibrinolytic activities correlated with the presence of two proteases in the extract, which were inhibited by 1,10-phenanthroline, indicating that the enzymes were metalloproteases (Ratel et al. 2005).

In conclusion, using a metagenomic approach, a novel protease gene was isolated. The enzyme exhibits fibrinolytic activity. Therefore, the metagenome may become a new source for fibrinolytic agents, and could be used to develop therapeutic agents for the treatment of thrombosis.

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