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RESEARCH PAPER

Molecular Cloning and Functional Expression of a Fibrinolytic Protease Gene from the Polychaeta, *Periserrula leucophryna*

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Abstract Full-length cDNA encoding a fibrinolytic protease (PLFP) from the cDNA library of the polychaete, *Periserrula leucophryna* was cloned, sequenced and expressed in *Escherichia coli*. The entire cDNA of the PLFP clone was 921 bp (CDS: 41-837), including a coding nucleotide sequence of 798 bp, a 5'-untranslaed region of 40 bp, and a 3'-noncoding region of 83 bp. The ORF encoded a 265-amino acid polypeptide precursor consisting of a 36-residue signal sequence and a 229-residue mature polypeptide. The sequence alignment results of PLFP revealed sequence similarity with several fibrinolytic enzymes. Sequence analysis revealed a conserved catalytic triad of His78, Asp126 and Ser219 residues, suggesting that PLFP is a serine protease. Mature PLFP had an apparent molecular weight of approximately 25 kDa and was produced in inclusion bodies when expressed in *E. coli*. Substrate specificity results that recombinant PLFP was active towards Arg-X or Lys-X and did not hydrolyze substrates with nonpolar amino acids at the P1 site. Recombinant PLFP was strongly inhibited by typical serine protease inhibitors, further indicating that PLFP is a member of the serine protease family. PLFP was able to dissolve artificial plasminogen-free fibrin, and its fibrinolytic behavior was similar to that of plasmin. Taken these results together, PLFP is a new member of the fibrinolytic enzyme family with selective specificity on fibrin, and the availability of PLFP offers an attractive alternative approach for thrombolysis therapy because rPLFP is believed to have advantages over currently used plasminogen activators, that is, lower price and lower side effect.

Keywords: cloning, expression, plasmin-like activity, *Periserrula leucophryna*

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1. Introduction

Blood coagulation disorders are global and frequently lethal diseases. When clots are not removed, they cause thrombosis, resulting in cardiovascular diseases [1,2]. Thrombosis can occur in any vessel in the body, and the principal clinical syndromes are cardiovascular diseases, such as acute myocardial infarction and acute stroke. Fibrinolytic enzymes are divided into two types: (1) plasminogen activators such as tissue-type plasminogen activator (t-PA) and urokinase (UK) [3-5], which activate plasminogen to plasmin and (2) plasmin-like proteases, which rapidly and directly dissolve fibrin clots. Although plasminogen activators are widely used [1-3,5], these enzymes are expensive, and patients may suffer undesirable side effects, such as internal hemorrhaging. Therefore, plasmin-like proteases have been purified and characterized from various sources, such as nattokinase from *Bacillus* sp. [6], fibrolase from the venom of *Agkistrodon contortrix* [7], atroxase from *Crotalus atrox* venom [8], lebetase from *Vipera lebetina* venom [9], lumbrokinase (LK) from earthworms [10-12], and fibrinolytic enzymes from insects [13-16], fungi [17,18], marine green alga [19,20] and some

bacteria [6,21-27] in order to increase the efficacy and specificity of thrombolytic therapies. Accordingly, it is desirable to explore new sources of plasmin-like proteases since few enzymes have been reported for practical thrombolytic applications. Recently, we described numerous proteins purified from the polychaeta, *Periserrula leucophryna*, which lives in the tidal flats of the Korean west sea (yellow sea), including a novel PLFP [28], ferritin [29] and cysteine protease [30]. Among these, the PLFP purified from the hemolymph of *P. leucophryna* shows strong plasmin-like activity, suggesting it is a good candidate for practical applications in thrombolysis.

In this study, we carried out the isolation and characterization of the PLFP gene from the cDNA library of *P. leucophryna*, and its deduced amino acid sequence was analyzed. We also measured PLFP expression using an *E. coli* expression vector under control of the T7 promoter [31,32] as well as the functions of recombinant PLFP (rPLFP). This is the first report on the identification, molecular cloning and expression of a novel serine protease exhibiting fibrinolytic activity from a polychaeta, *P. leucophryna*.

2. Materials and Methods

2.1. Bacterial strains, medium, and plasmids

E. coli BL21(DE3) strain which was kindly provided by the Waterborne Virus Bank in the Republic of Korea (http://knrrb.knrrc.or.kr) was utilized for expression of the T7 promoter system, specifically its non-toxic protein expression. The pGEM-Teasy vector (Promega, USA) was utilized for cloning of the PCR products. The pT7-7 plasmid [32] harboring the T7 promoter was used to construct the expression plasmids of the PLFP gene. *E. coli* XL1-Blue MRF' (*F', proAB, lacIqZΔM15, thi, recA, gyrA, relA, supE, Tn10*) was utilized as the host strain for construction of a cDNA library, and an *E. coli* SOLR strain (*F', proAB, lacIqZΔM15, lac, gyrA, relA, thi, sbcC, recB, Tn5, Su*$^-$) was utilized for *in vivo* excision. *E. coli* cells harboring plasmid were grown in Luria-Bertani (LB) medium supplemented with 50 μg of ampicillin/mL.

2.2. Protease assay and protein concentration

Protease activity was assayed with Val-Phe-Lys-pNA, according to a previously described method [33]. The reaction was carried out in a total volume of 200 μL of assay buffer (0.1 M Tris–HCl, pH 8.2) and an appropriate amount of enzyme. Ten microliters of 4 mM Val-Phe-Lys-pNA was added to the reaction mixture, and the enzyme reaction was allowed to proceed at 37°C for 2 min. The amount of para-nitroanilide (pNA) released was measured. Protein concentrations were determined with a Bio-Rad protein assay kit using bovine serum albumin as a standard [34].

2.3. Amplification of partial gene for screening the full-length PLFP gene

To obtain the partial gene fragment for screening the full-length PLFP cDNA, degenerated primers were designed and synthesized on the basis of the conserved sequences near (QHPGYNR) or within (CQGDSGG) the catalytic triad of serine protease [13]: 5'PLFP-F primer (5'CAR CAYCCIGGITAYAAYMG3') and 3'PLFP-R primer (5'CCI CCISWRTCICCYTBRCA 3'). Total cDNAs or RNA was used as the template for PCR. The DNA was amplified in a thermal cycler (Technique, USA) under the following conditions: 95°C 5 min, 50°C 1 min, 72°C 1 min (1 cycle); 95°C 1 min, 50°C 1 min, 72°C 1 min (29 cycles); 72°C 5 min. The PCR products were fractionated on 1% agarose gel, after which the DNA fragments were recovered and cloned into pGEM-Teasy vector. The proper clones were then screened and sequenced in both directions using M13 forward and reverse primers with an automated sequencer (Applied Biosystems, USA). Using BLAST search program, the partial gene was characterized to be a part of serine protease family and used as probe DNA to screen the full-length PLFP cDNA.

2.4. Screening of PLFP gene from *P. leucophryna* cDNA library

The full-length cDNA clones were screened *via* plaque hybridization using the *P. leucophryna* cDNA library constructed previously [30]. Approximately 5×10^5 plaques from the cDNA library per plate were utilized for the screening of the full-length cDNA. The partial PLFP gene (324 bp) was labeled using a Prime-a-Gene labeling kit (Promega, USA), and the unincorporated nucleotides were removed using a Sephadex G-50 spin column. The resultant probe was hybridized overnight to plaques on a colony/plaque screen filter (PerkinElmer, USA) at 42°C in 2x PIPES (0.8 M NaCl, 20 mM PIPES, pH 6.5), 50% deionized formamide, 0.5% SDS, and salmon sperm DNA (100 μg/mL). The positive phages were acquired after tertiary screening, and the pBluescript phagemid harboring the inserted DNA was excised using ExAssistTM helper phage and *E. coli* SOLR strain, in accordance with the manufacturer's instructions (Stratagene, USA).

2.5. Construction of expression vector containing T7 promoter

The sequence encoding the predicted mature PLFP gene was amplified from the obtained clone *via* PCR using an oligonucleotide primer set. The *Nde*I and *Hin*dIII restriction enzyme sites were added (underlined) to assist cloning into

the pT7-7 expression vector in the frame. The forward and reverse primers for PLFP were T7-PLFP-F (5' GGG GCATATGATTGTAGGAGGCCAGGACGCC3') and T7-PLFP-R (5' CCCCAAGCTTTTAGTTGATGTTTCCTATCC A3'), respectively. The mature form of the PLFP gene was amplified under the following conditions: 95°C 1 min, 50°C 1 min, 72°C 1 min (1 cycle); 95°C 1 min, 50°C 1 min, 72°C 1 min (29 cycles); 72°C 5 min (1 cycle). The PCR products were fractionated on 1% agarose gel, after which the DNA fragments were recovered, digested with *Nde*I and *Hin*dIII, cloned into linearized pT7-7 and named pT7-PLFP.

2.6. Protein induction and SDS-PAGE analysis

The recombinant expression vector harboring the active PLFP gene was transferred into *E. coli* BL21(DE3). When the cultures reached a cell density (A_{600}) 0.4 ~ 0.6, IPTG (isopropyl- β-D-thiogalactoside) was added to a final concentration of 1 mM. After an additional 6 h of incubation, the lysates were prepared from 1 mL of the culture. The cell pellets were then suspended in 200 μL of sample buffer (0.05 M Tris-HCl, pH 6.8, 0.1 M DTT, 2% SDS, 10% glycerol, and 0.1% bromphenol blue) and boiled for 5 min at 90°C. The samples (10 μL) were analyzed *via* SDS-PAGE, as described by Laemmli [35], using a mini-gel system (Bio-Rad, USA).

2.7. Refolding and purification of the recombinant PLFP

E. coli BL21(DE3) harboring pT7-PLFP was grown overnight at 37°C in LB medium supplemented with 50 μg of ampicillin/mL. The mixture (100 mL) was inoculated with 1 mL of the seed culture in 500-mL baffled flasks, followed by incubation at 37°C with shaking at 250 rpm. When the cell density reached an A_{600} value of 0.5, IPTG was added to a final concentration of 1 mM. After additional incubation for the indicated time, the cells were recovered by centrifugation (8,000 × *g*, 20 min). After washing briefly in 0.1 M Tris-HCl buffer, pH 8.2 (buffer A), cells were suspended in ice-cold identical buffer (5 mL/g of wet cells), sonicated (10 cycles of 30 sec each, with cooling for 5 min between cycles), and centrifuged for 30 min (12,000 × *g*, 4°C). The resulting pellet was washed twice with IB (inclusion body) washing buffer (buffer A containing 2.5 M urea and 2% Triton X-100) and centrifuged for 30 min (12,000 × *g*, 4°C). The pellet was then dissolved in denaturing buffer (buffer A containing 8 M urea, 10 mM NaCl, 2 mM EDTA and 1 mM DTT) for 4 h at 4°C with vigorously stirring. The insoluble material was removed by centrifugation for 30 min (20,000 × *g*, 4°C), and the supernatant was recovered. The protein was refolded by gradually removing urea *via* dialysis against buffer A with vigorous stirring. The rPLFP was purified by affinity chromatography on a Benzamidine-Sepharose column as described previously [28]. Briefly, the supernatant recovered by centrifugation was applied to a Benzamidine-Sepharose column (2.5 × 4 cm), which had been equilibrated with buffer A containing 0.5 M NaCl. The column was washed with the same buffer until the optical density of effluent at 280 nm almost reached zero, after which bound PLFP was eluted with buffer A containing 2 mM benzamidine at a flow rate of 20 mL/h. The active fractions were concentrated and then diafiltrated using Centriprep YM10 (Millipore, Bedford, USA) with buffer A to remove benzamidine, divided into aliquots, and stored at -70°C until use.

2.8. Substrate specificity

Purified rPLFP was assayed for amidolytic activity towards a variety of chromogenic protease substrates. The reaction was conducted in a total volume of 200 μL of assay buffer containing 50 ng of purified enzyme. Then, 10 μL of 4 mM stock of each substrate was added to the reaction mixture, and the reaction was allowed to proceed at 37°C for an additional 2 min. Proteolytic activity toward S-2251 was taken as a control.

2.9. Inhibition test

Purified rPLFP was pre-incubated with several inhibitors, such as leupeptin and PMSF (phenylmethylsulfonyl fluoride), for 30 min at room temperature, and the remaining activity was determined under standard assay conditions. Residual activities in the presence of inhibitors were compared to those of the controls without inhibitor.

2.10. Fibrinolytic activity

Fibrinolytic activity of purified rPLFP was assayed using the fibrin plate method according to Astrup and Müllertz [36]. Briely, 10 mL of 0.5% (w/v) human plasminogen-free fibrinogen solution in buffer A was poured into a petri dish (87 × 15 mm), after which 0.2 mL of bovine thrombin (10 NIH U/mL) was added. The mixture was then incubated for 30 min at room temperature to form fibrin clots. A 5-mm paper disc was carefully placed onto the fibrin plate, after which 10 μL of each sample was applied onto the disc, which was incubated for 4 h at 37°C. An equal volume of plasmin solution (1 U/mL) also was incubated as a control. Enzyme activity was estimated by measuring the width of the clear zone.

3. Results

3.1. Amplification of PLFP partial gene and sequence analysis

Using the designed primers synthesized on the basis of

the conserved sequences near (QHPGYNR) or within (CQGDSGG) the catalytic triad of serine protease, a partial 324 bp DNA fragment was obtained from total RNAs of *P. leucophryna* by RT-PCR. Sequencing identified it as a novel protease cDNA fragment having the same amino acid sequences in the conserved regions shown above. The deduced 108 amino acid sequence was compared to other sequences in the GenBank Protein databases using the BLAST program (NCBI, Bethesda, MD): fibrinolytic protease from *Perinereis aibuhitensis*, 53% (GenBank ACL12061.1); LK from *Lumbricus rubellus*, 42% (GenBank AAN28692.1); LK from *Lumbricus bimastus,* 42% (GenBank AAL28118.1); LK from *Eisenia fetida*, 42% (GenBank AAM73677.1). From these results, the amplified partial DNA fragment was characterized to be a fibrinolytic enzyme of serine protease family and used as a DNA probe to screen the full-length cDNA of PLFP.

3.2. Screening and characterization of full-length PLFP cDNA

Approximately 100,000 ~ 600,000 recombinant phage plaques were evaluated to obtain the full-length cDNA of PLFP using the cloned partial DNA fragment as a probe. More than 20 different clones were hybridized to the probe *via* primary, secondary and tertiary screening. Five plasmid clones from the hybridized phages were rescued and then digested with *Eco*RI and *Hin*dIII to confirm the size of inserted DNA (data not shown). The putative clones were analyzed by DNA sequencing. Finally, the confirmed PLFP gene consists of a 921 bp insert, which contained a 798 bp open reading frame (ORF: 41-837), a 5'-untranslaed region of 40 bp, and a 3'-noncoding region of 83 bp. Translation of the ORF generated a 265 amino acid polypeptide consisting of a 36-residue signal sequence and a 229-residue mature protein (Fig. 1). The molecular weight and pI value of the active PLFP were putatively calculated to be 24.4 kDa and 4.51, respectively. The conserved sequences near (QHPGYNR) or within (CQGDSGG) the catalytic triad of serine protease utilized for the synthesis of degenerate primers are underlined and the catalytic triads conserved in the serine proteases are represented as bold characters (His78, Asp126, and Ser219) in the boxes. As shown in Fig. 2, PLFP showed homology with other serine proteases, especially the fibrinolytic enzyme: 68% with fibrinolytic protease from *Perinereis aibuhitensis* (polychaeta, GenBank ACL12061.1); 47% with LK from *Lumbricus bimastus* (Earthworm, GenBank AAN78282.1); 45% with LK from *Eisenia fetida* (Red worm, GenBank ABQ23217.1); 44% with LK from *L. rubellus* (Earthworm, GenBank AAN28692.1); 43% with chymotrysin from *L. rubellus* (GenBank CAA11132.1); 41% with Trypsin-1 from *Camponotus floridanus* (carpenter ant, GenBank EFN68636.1),

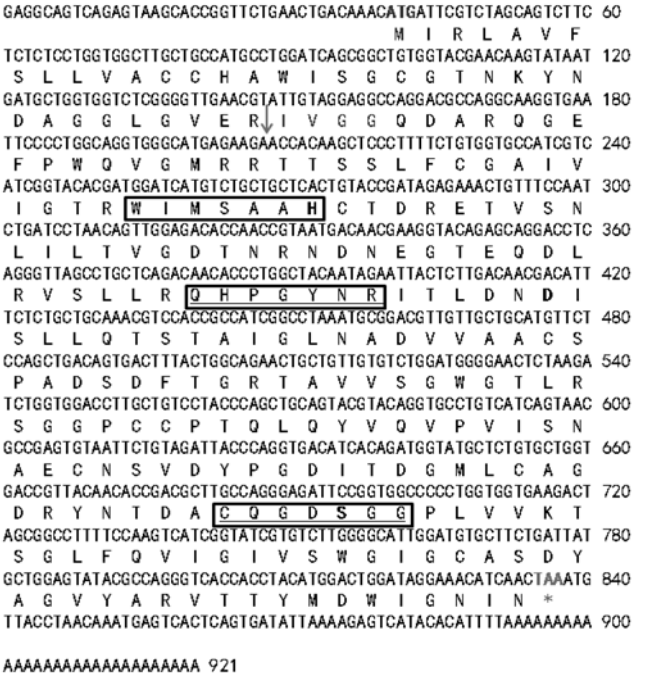

Fig. 1. Nucleotide (lower line) and deduced amino acid (upper line) sequences of PLFP gene from *P. leucophryna*. The PLFP gene consists of a 798-bp open reading frame (ORF: 41-816 bp). Translation of this ORF generated a 265-amino acid polypeptide comprising a 36-residue signal sequence and a 229-residue mature protein. The highly conserved amino acid residues among the serine proteases are shown in the boxes. The conserved sequences near (QHPGYNR) or within (CQGDSGG) the catalytic triad of serine protease utilized for synthesis of the degenerate primers are underlined in the boxes. Bold characters (His78, Asp126, and Ser219) indicate the active site, catalytic triad characteristics of the serine proteases. The arrow indicates cleavage site of peptidase. IVGG sequence means N-terminus of the mature PLFP form.

suggesting that PLFP is a fibrinolytic enzyme. In particular, the conserved catalytic triad (His78, Asp126 and Ser219) located in the active sites of PLFP is indicative of a trypsin-like serine protease (Fig. 2).

3.3. Expression and purification of recombinant PLFP

To express mature PLFP using the T7 promoter system in *E. coli*, the region of the mature form was amplified using two primers flanking each end of the PLFP gene. The forward primer had an *Nde*I restriction site harboring an initiation codon (AUG), whereas the reverse primer contained a termination codon (TAA) and a *Hin*dIII restriction site in order to facilitate cloning into the pT7-7 plasmid (Fig. 3). The resulting recombinant plasmid was transferred into *E. coli* BL21(DE3), after which expression of mature PLFP was induced for various time intervals at 37°C with 1 mM IPTG. The target protease with a molecular weight of approximately 25 kDa was produced in an inclusion body within 2 h, as detected by SDS-PAGE (Fig. 4) and

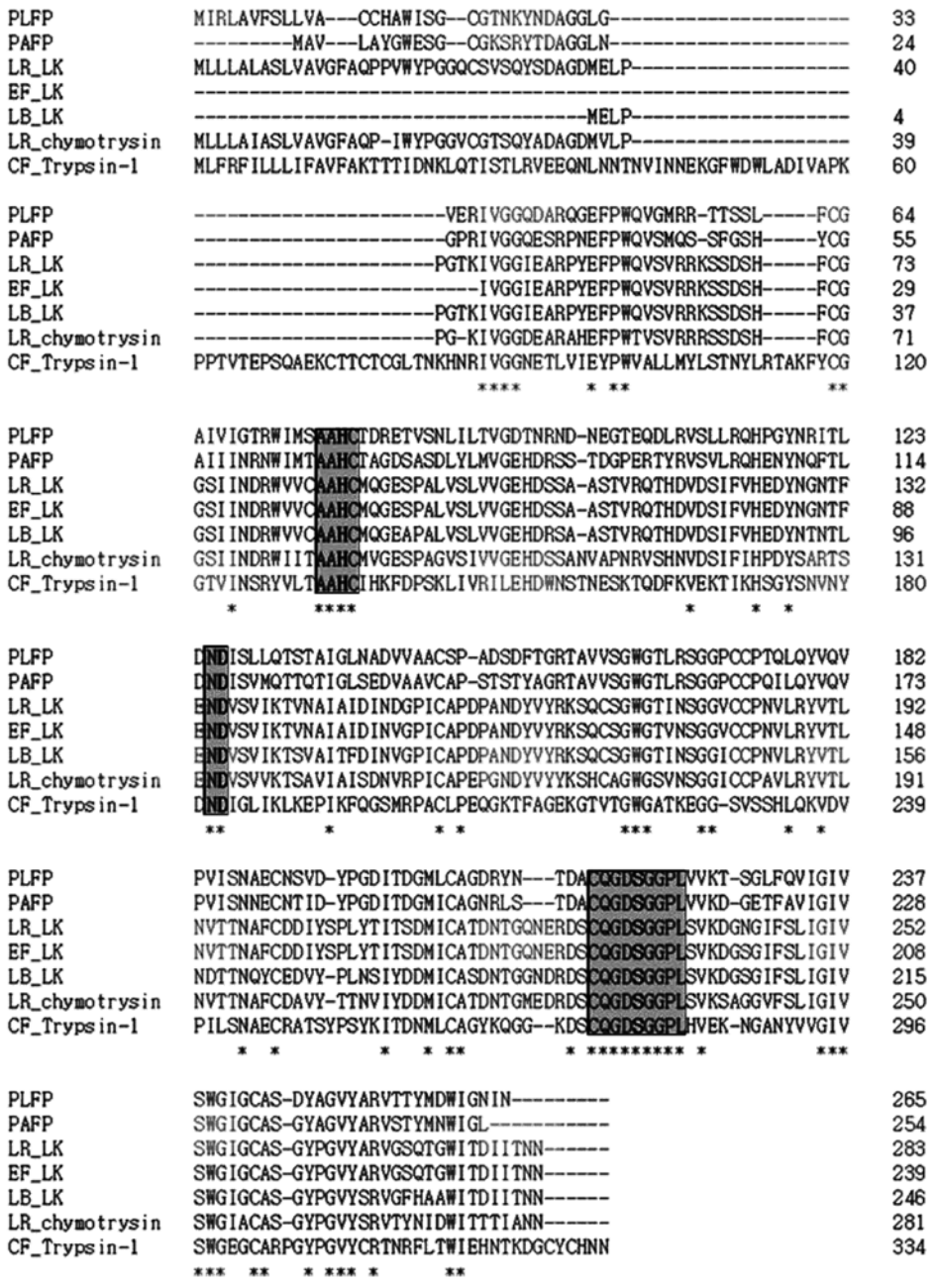

Fig. 2. Amino acid sequence alignment of serine proteases from different species**.** The shaded boxes indicate the conserved regions of the catalytic triad of serine proteases. PLFP, fibrinolytic protease from *P. leucophryna* (this study); PAFP, fibrinolytic protease from *Perinereis aibuhitensis* (GenBank ACL12061.1); LR_LK, lumbrokinase from *Lumbricus rubellus* (GenBank AAN28692.1); EF_LK, lumbrokinase from *Eisenia fetida* (GenBank ABQ23217.1); LB_LK, lumbrokinase from *L. rubellus* (GenBank AAN28692.1); LR_chymotrysin, chymotrysin from *L. rubellus* (GenBank CAA11132.1); CF_Trypsin-1, Trypsin-1 from *Camponotus floridanus* (GenBank EFN68636.1).

maintained its expression level even at 20 h. The partial amino acid sequence as analyzed by MALDI-MS indicated that the expressed protein was turned out to be the intact mature PLFP and N-terminal amino acids were started from IVGG (data not shown). To purify recombinant PLFP, the inclusion body was prepared using cells from 1 L of the culture, as described in Materials and Methods. After washing the pellet with IB washing buffer (Fig. 5, lane 4), the resulting inclusion bodies were dissolved in 40 mL of denaturing buffer and stirred vigorously for 6 h at 4ºC. Following recovery of the supernatant containing mature PLFP by centrifugation (20,000 × *g*, 4ºC, 30 min), refolding was carried out by gradually removing urea *via* dialysis. The supernatant containing soluble, refolded proteins (Fig. 5,

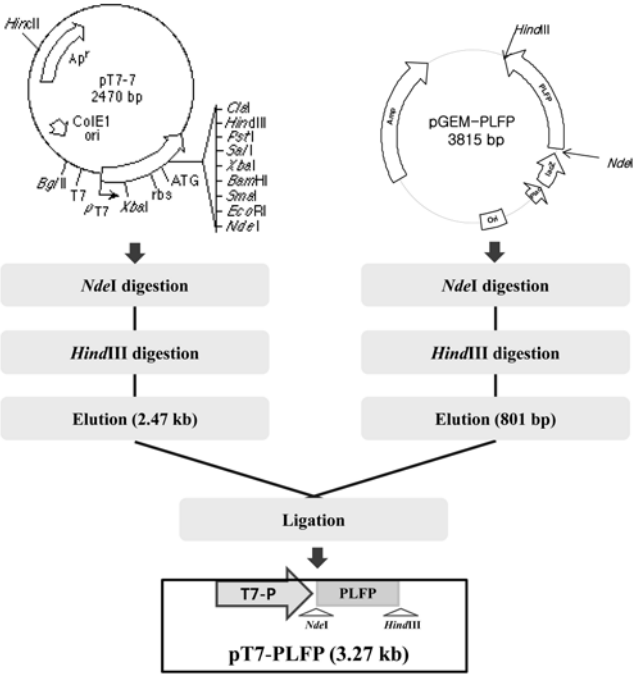

Fig. 3. Construction of expression plasmid for recombinant mature PLFP, pT7-PLFP.

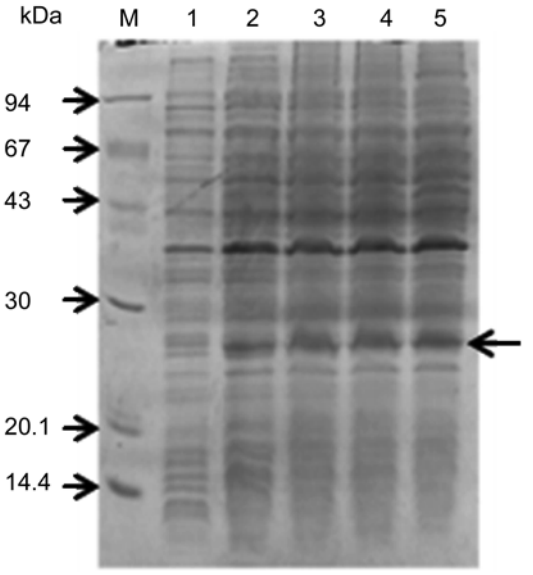

Fig. 4. Analysis of PLFP expression by SDS-PAGE. *E. coli* BL21(DE3) cells harboring pT7-PLFP were induced with 1 mM IPTG. Aliquots of the cell suspension (100 μL) were taken at regular time intervals, cells were recovered by centrifugation, and the resulting mixture was resolved by 15% SDS-PAGE. Lane M, molecular weight maker; lane 1, before induction; lanes 2 ~ 5, after induction with 1 mM IPTG for 2, 4, 6, and 20 h, respectively. Arrow indicated expressed PLFP. The molecular weight markers for SDS-PAGE are: phosphorylase b (94 kDa), BSA (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa).

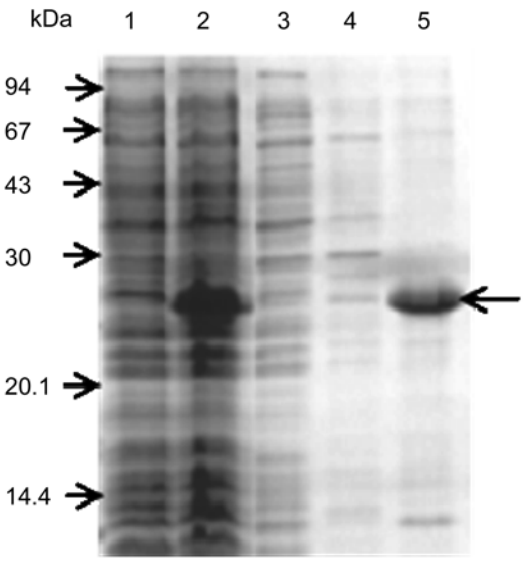

Fig. 5. Analysis of refolding process of PLFP expression by SDS-PAGE. Lane M, molecular weight maker; lane 1, before induction; lane 2, after induction with 1 mM IPTG for 20 h; lane 3, the supernatant fraction after sonication; lane 4, washing fraction from the pellet (inclusion body); lane 5, after refolding.

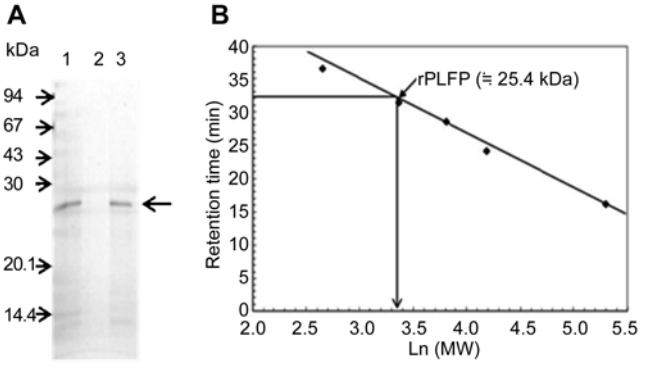

Fig. 6. Analysis of purified recombinant PLFP from inclusion bodies by SDS-PAGE (A) and FPLC on a Superose 12 HR (B). Panel A; lane 1, loading fraction (refolded rPLFP); lane 2, flow-through fraction from Benzamidine-Sepharose column; lane 3, purified rPLFP. The molecular weight markers for gel filtration were β-amylase (200 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), and α – lactalbumin (14.2 kDa).

lane 5) was collected by centrifugation (20,000 × *g*, 4ºC, 30 min), and then applied on affinity chromatography using Benzamidine-Sepharose column (2.5 × 4 cm), that was equilibrated with buffer A containing 0.5 M NaCl, followed by elution with buffer A containing 2 mM benzamidine. Purified rPLFP was found to be homogeneous, as analyzed by SDS-PAGE. The molecular weight of the protease was determined to be approximately 25 kDa by

SDS-PAGE (Fig. 6A) and FPLC on a Superose 12 HR column (Fig. 6B), indicating that rPLFP is a monomeric protein similar to native PLFP purified from the hemolymph of the polychaeta [28].

3.4. Substrate specificity of recombinant PLFP

The substrate specificity of purified rPLFP was evaluated using various synthetic substrates. The rPLFP showed higher activities towards Val-Phe-Lys-pNA and Tos-Gly-Pro-Lys-pNA, which are known to be specific substrates for plasmin (Table 1). Purified rPLFP also could hydrolyze Benz-Ile-Glu(γ-OR)-Arg-pNA, Pro-Phe-Arg-pNA, Val-Leu-Arg-pNA and Ile-Pro-Arg-pNA, whereas it was less active towards Val-Phe-Lys-pNA and Tos-Gly-Pro-Lys-pNA. Conversely, rPLFP could not efficiently hydrolyze substrates containing non-polar amino acids in the cleavage site, such as CBZ-Gly-Gly-Leu-pNA, Suc-Ala-Ala-Ala-pNA, and Suc-Ala-Ala-Pro-Phe-pNA (Table 1). The rPLFP which was more active towards substrates containing a basic amino acid at the P1 position, had a specificity similar to those of fibrinolytic enzymes, such as native PLFP [28], scolonase from *Scolopendra subspinipes mutilans* [23], fibrinolytic enzyme from venom of *Agkistrodon halys* [37] and fibrinolytic enzymes from *Eisenia fetida* [38].

3.5. Inhibition profile of recombinant PLFP

The effects of several natural and synthetic protease inhibitors on purified rPLFP were also assessed using Val-Phe-Lys-pNA as a substrate (Table 2). The enzymatic activity of rPLFP was very sensitive to PMSF and leupeptin, which inhibited greater than 90% of enzyme activity even at a very low concentration (0.2 mM). Other serine protease inhibitors including soybean trypsin inhibitor and benzamidine, partially inhibited rPLFP activity. In addition, the rPLFP activity was inhibited by approximately 80% by the trypsin selective reagent TLCK (0.5 mM); however, the chymotrypsin-alkylating agent TPCK (0.5 mM) did not inhibit enzyme activity. On the contrary, neither a metal ion chelator (EDTA) nor cystein protease inhibitors (cystatin, E-64) inhibited the activity of rPLFP. These results confirm that rPLFP is considered a member of the trypsin-like serine protease family, similar to PLFP purified from the hemolymph of polychaeta [13]. Similar inhibition profiles have been reported for other fibrinolytic enzymes including LK from *Eisenia fetida* [38], fibrinolytic serine protease from *Nereis virens* [39], plasmin-like protease from *Tenodera sinensis* [14], scolonase from *Scolopendra subspinipes mutilans* [13] and fibrinolytic enzyme 1 from *Fomitella fraxinea* [40].

3.6. Fibrinolytic activity of recombinant PLFP

Fibrinolytic activity of rPLFP was examined by measuring the clear zone of inhibition in an artificial fibrin plate assay. Purified rPLFP exhibited a large clear zone of inhibition similar to that of plasmin, which was used as a positive control (Fig. 7, well 1), following treatment with 1 μg of purified rPLFP (Fig. 7, well 2). However, trypsin (1 μg) as

Table 1. Substrate specificity of purified recombinant PLFP from inclusion bodies

Substrates	Protease	Relative activity (%)
Val-Phe-Lys-pNA	Plasmin	100.0[a]
Tos-Gly-Pro-Lys-pNA	Plasmin	92.9
Benz-Ile-Glu(γ-OR)-Arg-pNA	Factor Xa	87.1
Pro-Phe-Arg-pNA	Kallikerein	80.6
Val-Leu-Arg-pNA	Serine protease	48.4
Ile-Pro-Arg-pNA	t-PA	48.4
CBZ-Gly-Gly-Leu-pNA	Subtilisins	n.d.
Suc-Ala-Ala-Ala-pNA	Elastase	n.d.
Suc-Ala-Ala-Pro-Phe-pNA	Chymotrypsin	n.d.

[a]The activity toward Val-Phe-Lys-pNA was defined as 100%. n.d., not detected.

Table 2. Effects of various inhibitors on the activity of purified rPLFP

Inhibitor	Concentration	Remaining activity (%)*
None	-	100.0
Benzamidine	1 mM	32.2
Leupeptin	0.2 mM	9.3
	1 mM	6.0
PMSF	0.2 mM	3.5
	1 mM	1.2
TLCK	0.5 mM	21.3
TPCK	0.5 mM	93.3
SBTI	5 μg	33.6
Cystatin	5 μg	95.9
Chymostatin	5 mM	89.1
E-64	1 mM	98.6
EDTA	1 mM	101.5

*Ten nanogram of purified rPLFP was preincubated with inhibitors for 30 min at room temperature, after which 10 μL of chromogenic substrate (4 mM Val-Phe-Lys-pNA) were added. Residual activity was measured after incubation at 37°C for 5 min.

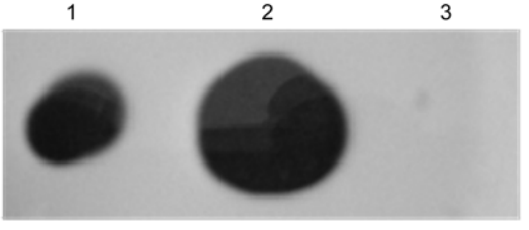

Fig. 7. Fibrinolytic activity of purified rPLFP on a plasminogen-free fibrin plate. Each sample (10 μL) was applied after the 5 mm-paper disc was carefully positioned on the fibrin plate and incubated for 4 h at 37°C. (1) Plasmin (10 mU); (2) purified rPLFP (1 μg); and (3) Trypsin (1 μg).

a negative control did not produce a clear zone of inhibition (Fig. 7, well 3). These results indicate that rPLFP is a plasmin-like protease with strong fibrinolytic activity similar to that of nattokinase from *Bacillus* species [6], fibrolase from snake venoms [7] and LK from earthworms [12].

4. Discussion

In this study, we describe the cloning of a fibrinolytic enzyme, designated as PLFP, its potential for practical applications in thrombolytic therapy and its expression in *E. coli* BL21(DE3).

This is the first report on the identification, molecular cloning and expression of a novel serine protease exhibiting fibrinolytic activity from a polychaeta, *P. leucophryna*. The characteristics of the isolated PLFP cDNA were follows: (1) The entire cDNA of PLFP clone was 921 bp (CDS: 41-837), including a coding nucleotide sequence of 798 bp, a 5'-untranslaed region of 40 bp, and a 3'-noncoding region of 83 bp; (2) the ORF of PLFP was a 265 amino acid polypeptide consisting of a 36-residue signal sequence and a 229-residue active protein; (3) the N-terminal region of mature PLFP, starting from IVGG sequence was 100% identical to the native PLFP purified from the hemolymph of *P. leucophryna*; (4) it showed high sequence homology with the fibrinolytic enzyme from *Perinereis aibuhitensis* and LK from *Lumbricus bimastus* and *Eisenia fetida*; (5) the rPLFP expressed in *E. coli* BL21(DE3) was effectively refolded into an active soluble protein; (6) biochemical characteristics of rPLFP expressed in *E. coli* BL21(DE3) were similar to those of native PLFP purified from the hemolymph of *P. leucophryna,* specifically, an apparent molecular weight of 25 kDa, serine protease activity (substrate specificity and inhibition profile) and strong fibrinolytic activity.

Although plasminogen activators including t-PA and UK are widely used currently, these enzymes exhibit some weak points such as high price, low fibrin specificity, allergic reactions, and undesirable side effects such as internal hemorrhaging [41,42]. Therefore, continuous efforts to search new type of safer and less expensive fibrinolytic enzymes which rapidly and directly act on fibrin clots have been concentrated from diverse sources including earthworms, snake venoms and microorganisms [6-12].

Taken these results together, the biochemical properties of rPLFP expressed in *E. coli* BL21(DE3) are similar to those of plasmin-like enzymes, such as LK from earthworms and nattokinase from *Bacillus* species [6], and a new member of the fibrinolytic enzyme family with selective specificity on fibrin. The availability of this fibrinolytic enzyme offers an attractive alternative approach for thrombolysis therapy because rPLFP is believed to have advantages over currently used plasminogen activators, that is, low price and low side effect.

Abbreviations

EDTA : Ethylene diamine tetraacetic acid
FPLC : Fast protein liquid chromatography
IPTG : Isopropyl- β-D-thiogalactoside
LB : Luria-Bertani
LK : Lumbrokinase
ORF : Open reading frame
PA : Plasminogen activator
PLFP : *Periserrula leucophryna* fibrinolytic protease
PMSF : Phenylmethylsulfonyl fluoride
rPLFP : Recombinant PLFP
t-PA : Tissue-type plasminogen activator
TLCK : Nα-tosyl-L-lysine chloromethyl ketone
TPCK : Nα-tosyl-L-phenylalanine chloromethyl ketone
UK : Urokinase

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