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Purification, Characterization, and Cloning of Fibrinolytic Metalloprotease from *Pleurotus ostreatus* Mycelia

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Abstract A fibrinolytic protease (PoFE) was purified from the cultured mycelia of the edible oyster mushroom *Pleurotus ostreatus*, using a combination of various chromatographies. The purification protocol resulted in an 876-fold purification of the enzyme, with a final yield of 6.5%. The apparent molecular mass of the purified enzyme was estimated to be 32 kDa by SDS-PAGE, fibrin-zymography, and size exclusion using FPLC. The optimal reaction pH value and temperature were pH 6.5 and 35°C, respectively. PoFE effectively hydrolyzed fibrinogen, preferentially digesting the Aα-chain and the Bβ-chain over the γ-chain. Enzyme activity was enhanced by the addition of Ca^{2+}, Zn^{2+}, and Mg^{2+} ions. Furthermore, PoFE activity was potently inhibited by EDTA, and it was found to exhibit a higher specificity for the chromogenic substrate S-2586 for chymotrypsin, indicating that the enzyme is a chymotrypsin-like metalloprotease. The first 19 amino acid residues of the N-terminal sequence were ALRKGGAAALNIYSVGFTS, which is extremely similar to the metalloprotease purified from the fruiting body of *P. ostreatus*. In addition, we cloned the PoFE protein, encoding gene, and its nucleotide sequence was determined. The cDNA of cloned *PoFE* is 867 nucleotides long and consists of an open reading frame encoding 288 amino acid residues. Its cDNA showed a high degree of homology with PoMEP from *P. ostreatus* fruiting body. The mycelia of *P. ostreatus* may thus represent a potential source of new therapeutic agents to treat thrombosis.

Keywords: *Pleurotus ostreatus*, mushroom, mycelia, fibrinolysis, metalloprotease

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Fibrinolytic enzymes dissolve the blood clots, which are formed by the conversion of fibrinogen into fibrin *via* the proteolytic action of thrombin [48]. The insoluble fibrin fiber is hydrolyzed into fibrin degradation products by plasmin, which is generated from plasminogen by plasminogen activators such as the tissue plasminogen activator, vascular plasminogen activator, blood plasminogen activator, urokinase, Hageman factor, and streptokinase-plasminogen complex [12, 13]. Fibrin clot formation and fibrinolysis are normally well balanced in biological systems. However, when fibrin is not hydrolyzed because of some disorder, thromboses can occur. Myocardial infarction is the most common of these thromboses. Cardiovascular diseases, including acute myocardial infarction, are the leading causes of death throughout the world [37].

The fibrinolytic agents available today for clinical use are mostly plasminogen activators such as a tissue-type plasminogen activator (tPA), a urokinase-type plasminogen activator, and the bacterial plasminogen activator streptokinase. Despite their widespread use, all these agents have undesired side effects, exhibit low specificity for fibrin, and are also relatively expensive. Therefore, the searches for other fibrinolytic enzymes from various sources are being continued. Over the last decade, potent fibrinolytic enzymes have been discovered from a variety of sources, such as earthworms [36, 49], snake venoms [16, 23], insects [2], food-grade microorganisms [1, 5, 22, 25], marine creatures [44], herbal medicines [9], and fermented food products like Japanese *natto* [11, 42, 44], Korean *chungkook-jang* [30], and Chinese *douchi* [48].

In recent years, mushrooms have become an attractive source of various physiologically active compounds [50, 53]. They are commonly used as food and food flavoring

substances and also in traditional oriental medicines. Their extracts have been reported to exert hematological, antiviral, antitumorigenic, hypotensive, and hepatoprotective effects [6, 7, 21]. They constitute an important source of thrombolytic agents. Many fibrinolytic enzymes were identified in the fruiting body of different medicinal mushrooms, such as *Armillaria mellea* metalloprotease (AmMEP) from *Armillaria mellea* [20, 26], *Grifora frondosa* aminopeptidase from *Grifola frondosa* [38], *Pleurotus ostreatus* metalloprotease (PoMEP) from *Pleurotus ostreatus* [8, 24], and two novel fibrinolytic proteases from *Fomitella fraxinea* [33]. All of these enzymes are Zn-metalloprotease group proteins and belong to the same wood-rotting fungi. Indeed, the presence of fibrinolytic enzymes in the fruiting body of some mushrooms has been identified, although the presence of these enzymes in their cultured mycelia is not clear. Furthermore, protease genes from several bacteria, fungi, and viruses have been cloned and sequenced with the prime aims of overproduction of the enzyme, delineation of the role of the enzyme in pathogenicity, and alteration in enzyme properties to suit its commercial application. Despite the extensive research on several aspects of proteases, there is a paucity of knowledge about the genetic analysis of fibrinolytic enzymes. Therefore, we have attempted to find fibrinolytic enzymes in *P. ostreatus* as well as other medicinal mushrooms. Recently, we purified and characterized a fibrinolytic enzyme from *Cordyceps militaris* [28] and cultured mycelia of *Armillaria mellea* [35]. In this study, we describe the purification, characterization, and cloning of a fibrinolytic enzyme from the mycelia of edible oyster mushroom, *P. ostreatus*.

MATERIALS AND METHODS

Materials

For the protein purification and characterization, the *Pleurotus ostreatus* (*P. ostreatus*) strain was supplied from the Department of Industrial Crop Production and Processing, Iksan National College, Republic of Korea. Human fibrinogen, human thrombin (1,000 units), plasmin (10 units), azocasein, phenylmethyl sulfonylfluoride (PMSF), *N*-α-tosyl-L-lysine chloromethyl ketone (TLCK), *N*-α-tosyl-L-phenylalanine chloromethyl ketone (TPCK), and 4-amidinophenylmethane sulfonylfluoride (APMSF) were purchased from the Sigma-Aldrich Co. (U.S.A.). DEAE Sephadex A-50, Sephadex G-75, and HiLoad 16/60 Superdex 75 were purchased from Pharmacia Biotech (Sweden). ProSieve color protein marker and ProSieve protein marker were purchased from Cambrex Co. (U.S.A.). Chromogenic subtrates were purchased from Chromogenix Co. (Sweden). An other chemicals were purchased from Sigma-Aldrich Co. (U.S.A.). Other reagents were special grade, and were purchased commercially. The TA cloning vector pGEM-T easy and TriZol reagent were purchased from Promega and Life Science, respectively. Reverse transcriptase (M-MLV), Taq DNA polymerase, and T4 DNA ligase were purchased from Bioneer Co. (Republic of Korea).

Cultivation of *P. ostreatus* Mycelia

The stock culture of *P. ostreatus* was maintained on PDA slants. The slants were inoculated with mycelia and incubated at 25°C for 7 days, and then used for seed culture inoculation. The mycelia of *P. ostreatus* were transferred to the seed culture medium by punching out about 5 mm^2 of the slants with a sterilized cutter. The seeds were inoculated into 500-ml flasks containing 200 ml of synthetic medium (40 g glucose, 10 g yeast extract, 0.5 g KH_2PO_4, 0.5 g $K_2HPO_4 \cdot 3H_2O$, and 0.5 g $MgSO_4 \cdot 7H_2O$ per liter) and incubated at 25°C on a rotary shaker at 110 rpm for 5 days.

General Protease Assay

Protease activity was determined by measuring the release of acid-soluble material from azocasein (Sigma). Enzyme sample/column fraction (50 μl) was added to 300 μl of 1% (w/v) azocasein (prepared in 50 mM Tris-HCl, pH 7.0). Following the incubation at 37°C for 20 min, 600 μl of ice-cold 10% (w/v) trichloroacetic acid was added with simultaneous vortexing. The sample was placed on ice for 10 min before centrifugation at 15,000 rpm for 15 min. The quantity of acid-soluble material in the supernatant was measured by absorbance at 366 nm. One unit of protease activity was defined as the amount required to produce enough acid-soluble material from azocasein to yield an absorbance of 0.1 at 366 nm, following 1 h of incubation at 37°C.

Purification of Fibrinolytic Enzyme

All procedures were carried out at 4°C. *P. ostreatus* mycelia were collected by centrifugation at 600 ×*g* and immediately stored in a −70°C freezer. Frozen mycelia were thawed and homogenized with an equal volume of water in a Kenwood blender for 2 min at maximum speed. The homogenate was centrifuged at 600 ×*g* and 4°C for 30 min. The crude extract was placed on ice. An equal volume of pre-chilled ethanol was added, dropwise, with constant stirring, after which the solution was kept stirring for a further 1 h. Precipitated protein was removed by centrifugation at 600 ×*g* for 30 min at 4°C. The clarified ethanol-soluble fraction was returned to the ice. Its ethanol concentration was increased, dropwise, to 70% with constant mixing. Stirring was continued for 1 h, after which precipitated protein was recovered by centrifugation at 600 ×*g* for 30 min and 4°C. Following removal of the supernatant the pellets were dried and the protein was then resuspended in optimal buffer for ion-exchange chromatography. Insoluble material was removed by

centrifugation at 10,000 ×*g* for 10 min at 4°C. The resuspended pellets were first applied to a CM-cellulose column (3.5×10 cm) equilibrated with 10 mM citrate-NaOH buffer (pH 6.0) and eluted with a linear gradient of 0–1.0 M NaCl at a flow rate of 1.0 ml/min at 4°C. The active fractions were pooled and then applied to a DEAE Sephadex A-50 column (3.5×10 cm) equilibrated with the 20 mM Tris-HCl buffer (pH 8.0), and eluted with a linear gradient of 0 to 1.0 M NaCl (pH 7.4) at a flow rate of 1.0 ml/min at 4°C. Active fractions were pooled and concentrated by freeze-drying and desalting. In order to further purify the sample, gel filtration was performed with a Sephadex G-75 column (1.5×100 cm) in 0.05 M phosphate buffer containing 0.15 M NaCl (pH 7.2) at a flow rate of 0.1 ml/min. Active fractions were collected and concentrated by freeze-drying. Desalted active faction was dissolved in 0.05 M phosphate buffer containing 0.15 M NaCl (pH 7.4) for FPLC on a HiLoad 16/60 Superdex 75 column (Amersham Bioscience Co.). Further fractionation was done using a HiLoad 16/60 Superdex 75 column equilibrated with 0.05 M phosphate buffer containing 0.15 M NaCl (pH 7.4) at a flow rate of 1.0 ml/min. Active fractions were pooled and concentrated by a Centricon 30,000 (Amicon Co. U.S.A.), and analyzed for purity by SDS-PAGE.

Molecular Mass Determination

The molecular mass of the enzyme was determined by SDS–PAGE, fibrin-zymography, and FPLC using a HiLoad 16/60 Superdex 75. SDS-PAGE was carried out according to the methods described by Laemmli [32] using 12% polyacrylamide gel, and the gel was stained with Coomassie brilliant blue R-250. Fibrin-zymography was carried out according to the methods of Kim *et al.* [29]. Resolving gel solution (12%) containing 0.12% (w/v) fibrinogen was prepared in a total 10 ml volume, and then centrifuged to remove insoluble impurities that were introduced when the SDS stock solution was mixed. Thrombin (1 unit/ml) solution and *N,N,N,N*-tetramethylethylenediamine (TEMED) were added to the gel solution in final concentrations of 0.1 μunit/ml and 0.028% (v/v), respectively. Purified enzymes were electrophorized on fibrin gel, washed in 2.5% Triton X-100 solution, and incubated in a bath containing reaction buffer (prepared 20 mM Tris-HCl, pH 7.5, containing 0.15 M NaCl, 0.02% NaN_3) at 37°C for 12–15 h. Gel filtration chromatography was performed at room temperature, using a HiLoad 16/60 Superdex 75 column (ΔKTA FPLC) for the estimation of the molecular mass of the enzyme at a flow rate of 1.0 ml/min. A gel filtration protein marker comprising glyceraldehyde-3-phosphate dehydrogenase (35 kDa), carbonic anhydrase (29 kDa), trypsinogen-PMSF (24 kDa), and trypsin inhibitor (20.1 kDa) was used.

Determination of N-Terminal Sequence

The N-terminal amino acid sequence of purified fibrinolytic enzyme was determined using an Applied Biosystems Precise 491 amino acid sequencer at the Korea Basic Science Center in Seoul. Sequenced data and sequence alignment were analyzed using Blast in the NCBI protein database and default parameters.

Fibrinolytic and Fibrinogenolytic Assays

Fibrinolytic activity was determined using the method described by Ästrup and Mullertz [3], with minor modifications, as follows. The fibrin agarose plate was made to a 1-mm thickness, and contained 1.2% agarose, 0.4% human fibrinogen, and 20 units/ml of human thrombin. The clot was allowed to stand for 1 h at room temperature. Then, 10 μl of sample solution was carefully placed onto the plate. The plate was incubated for 5 h at 37°C and the diameter of the lytic circle was measured. In the fibrin plate method, a clear transparent region is observed in which fibrin is hydrolyzed, and its diameter is directly proportional to the potency of the fibrinolytic activity.

In addition, fibrin degradation analysis was performed by a slightly modified method of Datta *et al.* [14]. In brief, 10 μg of human fibrinogen solution (prepared 20 mM Tris-HCl, pH 7.5, containing 0.15 M NaCl) was added to human thrombin (0.1 NIH unit), and then allowed to stand for 1 h at room temperature. Formed clots were mixed with purified enzyme and incubated at 37°C for various time intervals. Plasmin was used as a positive control. The resulting enzymes were analyzed by SDS-PAGE on 12% gel. Fibrinogenolytic activity was measured as follows: 80 μl of 1.0% human fibrinogen (prepared 20 mM Tris-HCl, pH 7.5, containing 0.15 M NaCl) was incubated with 10 μg of a purified enzyme at 37°C. At various intervals, a portion of the reaction solution was withdrawn and analyzed by SDS-PAGE according to the method of Laemmli [32]. Plasmin was used as a positive control.

Effect of Temperature and pH on Enzyme Activity

The optimal temperature for activity of purified enzyme was determined by measuring residual activity after the incubation of 10 μl of purified enzyme in 90 μl of 20 mM Tris-HCl (pH 7.5) at different temperatures (20–90°C) for 1 h. The optimal pH for the fibrinolytic activity of the purified enzyme was determined within a pH range of 2–10. Ten μl of the enzyme solution was added to 90 μl of 0.5 M glycine-HCl (pH 2.0–3.0), 0.5 M acetate (pH 4.0–5.0), 0.5 M Tris-HCl (pH 6.0–8.0), and 0.5 M glycine-NaOH (pH 9.0–10.0) buffers. After 1 h of incubation at room temperature, the remaining protease activity of each enzyme solution was measured with 1% azocasein.

Effect of Metal Ions and Protease Inhibitors on the Enzyme Activity

The effects of metal ions were investigated using $MgCl_2$, $MnSO_4$, $ZnCl_2$, $CoCl_2$, $FeCl_2$, $CaCl_2$, and $CuSO_4$. The purified enzymes were pre-incubated in both the absence and the presence of bivalent cations, including Mg^{2+}, Ca^{2+}, Co^{2+}, Cu^{2+}, Zn^{2+}, Mn^{2+}, and Fe^{2+}, with a final concentration of 1.0 mM in 10 mM Tris-HCl (pH 7.4) for 1 h at 37°C. After 1 h of incubation at room temperature, residual protease activity was measured with 1% azocasein. The effects of protease inhibitors were also assessed, using ethylenediaminetetraacetic acid (EDTA), phenylmethylsulfonyl fluoride (PMSF), *N*-α-tosyl-L-lysine chloromethyl ketone (TLCK), *N*-α-tosyl-L-phenylalanine chloromethyl ketone (TPCK), and 4-amidinophenylmethane sulfonyl fluoride (APMSF), aprotinin, and pepstatin A at 37°C for 1 h. The residual enzyme activity was determined using 0.1% azocasein.

Amidolytic Activity of the Enzyme

Amidolytic activities were measured spectrophotometrically, using synthetic chromogenic substrates such as S-2222 (Bz-Ile-Glu-(OR)-Gly-Arg-*p*NA for factor Xa), S-2288 (H-D-Ile-Pro-Arg-*p*NA for tPA), S-2238 (H-D-Phe-Pip-Arg-*p*NA for thrombin), S-2251 (H-D-Val-Leu-Lys-*p*NA for plasmin and SK), S-2444 (pyroGlu-Gly-Arg-*p*NA for U.K.), S-2586 (MeO-Suc-Arg-Pro-Tyr-*p*NA·HCl for chymotrypsin) and S-2765 (Z-D-Arg-Gly-Arg-*p*NA·2HCl for factor Xa). Activities were evaluated by the mixing of 1 μg of purified enzyme with 300 μl of a 0.5 mM synthetic chromogenic substrate. After continuous measurement for 5 min at 37°C with a temperature-regulated spectrophotometer, the amount of released *p*-nitroaniline was determined by measuring the change in 405 nm.

cDNA Cloning and Sequence Analysis

Total RNA was isolated from mycelia of *P. ostreatus*, using the TriZol reagent, and then transcribed with MMLV-RT using oligo(dT) primer. The resulting cDNA was amplified by PCR with two primers (sense primer, 5'-GGATCCATGTTGCGCTCC ATCCTGTTAATTG-3'; antisense primer, 5'-AAGCT TTTACACTGGTGCTGCTAC CCTGGC-3'), which were specially designed according to the nucleotides sequence of PoMEP isolated from *P. ostreatus* fruit body (Geneank Accession No. AY640032). The PCR product was electrophorized on the agarose gel and then gel-eluted by a QIAavic Gel extraction kit (Qiagen, U.S.A.) and subcloned into pGEM-T easy vector. The recombinant plasmid was transformed into DH5α-competent cell and then amplified, purified by a QIA prep spin Miniprep kit (Quiagen, U.S.A.), and subjected to DNA sequence analysis. The N-terminal amino acid sequence of purified fibrinolytic enzyme was determined using an Automatic DNA sequencer (ABI PRISM 377, Perkin Elmer, U.S.A.) at the Korea Basic Science Center in Gwang-ju, Republic of Korea. Sequenced data and sequence alignment were analyzed using Blast in the NCBI protein database and default parameters.

RESULTS

Enzyme Purification and Molecular Mass Determination

The fibrinolytic enzyme was purified by the combination of a variety of chromatographic steps, listed in Table 1. The crude extract was subjected to cation-exchange chromatography on CM-cellulose proteins. The fibrinolytic enzyme peak is the single one in Fig. 1A. These fractions were collected and applied onto DEAE Sephadex A-50 and the active fractions were obtained (Fig. 1B). The active fractions were further separated *via* gel filtration chromatography on the Sephadex G-75 (Fig. 1C). The major fractions with fibrinolytic activity were collected and applied onto the HiLoad 16/60 Superdex 75 column using ΔCTA fast FPLC, which yielded one major peak showing strong fibrinolytic activity (Fig. 1D). As summarized in Table 1, the enzyme was purified 876-fold, with a final yield of 6.5% after these purification steps.

The molecular mass of the fibrinolytic enzyme from *P. ostreatus* was found to be 32 kDa, as estimated by size exclusion on HiLoad 16/60 Superdex 75 column, using ΔCTA fast FPLC (Fig. 2A). This value is similar to the value estimated by SDS-PAGE and fibrin-zymography (Fig. 2B).

N-Terminal Amino Acid Sequence of Purified Enzyme

The N-terminal amino acid sequence of the purified fibrinolytic enzyme from *P. ostreatus* was analyzed via automated

Table 1. Purified fibrinolytic metalloprotease activity yields from mycelia of *P. ostreatus*.

Purification step	Volume (ml)	Total protein (mg)	Proteolytic activity (Unit)	Specific activity (Unit/mg)	Recovery (%)	Purification fold
Homogenate	250	N. D.	N. D.	-	-	-
Crude extracts	500	6,782	8,424	1.2	100	1
DEAE Sephadex A-75	5	3.98	1,573	395	18.7	329
Sephadex G-75	3	0.91	635	697.8	7.5	581.5
HiLoad 16/60 Superdex 75 pg	2	0.52	548	1,053	6.5	876

N.D., not determined. Protease activity was measured by using the azocasein assay, as described under Materials and Methods.

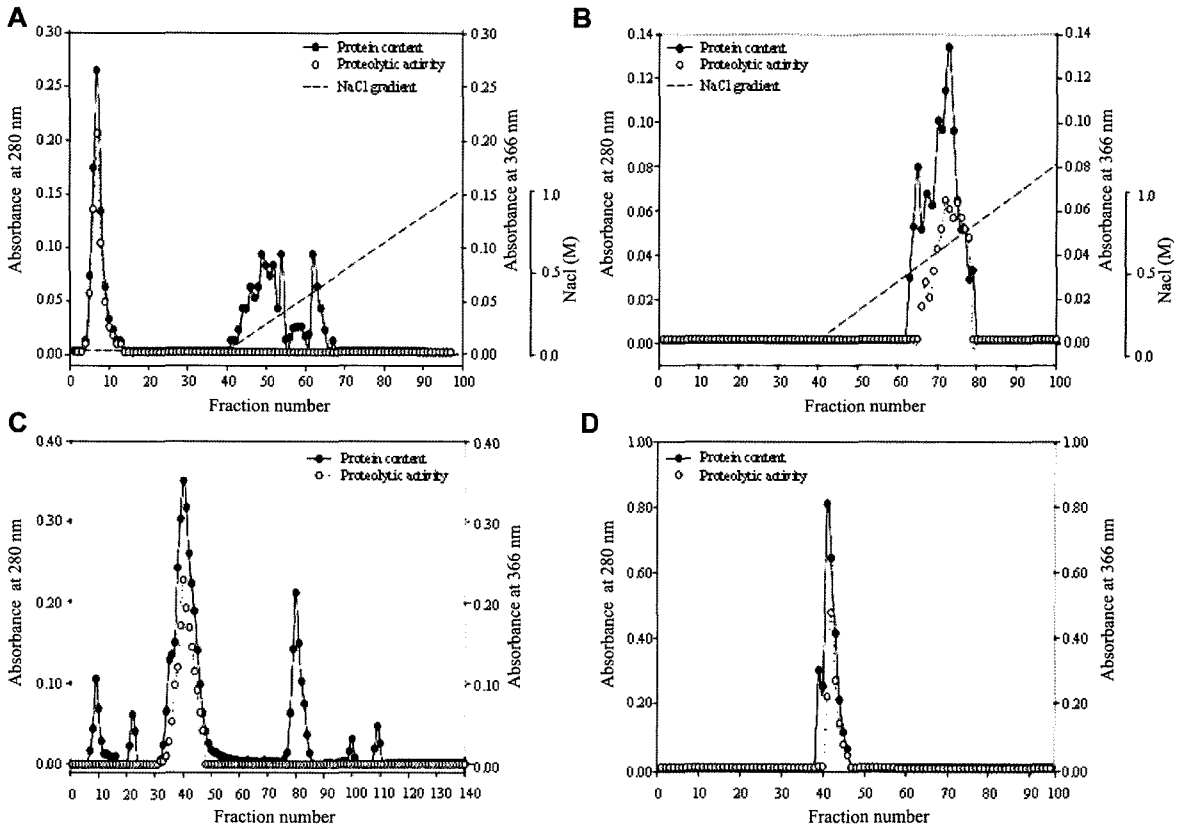

Fig. 1. Purification of fibrinolytic metalloprotease from *P. ostreatus*.
A. Cation-exchange chromatography on CM-cellulose column; **B**. Anion-exchange chromatography on DEAE-Sephadex A-50 column; **C**. Gel filtration on Sephadex G-75 column; **D**. HiLoad 16/60 Superdex 75 column. The elution profiles were monitored by spectrophotometry at 280 nm. Fibrinolytic activity was measured by azocasein assay at 366 nm.

Edman method, after SDS-PAGE and electroblotting. The N-terminal sequence of the first 19 residues was ALRKGGAAALNIYSVGFTS (Fig. 3) which is similar to that of the PoMEP from the *P. ostreatus* fruiting body

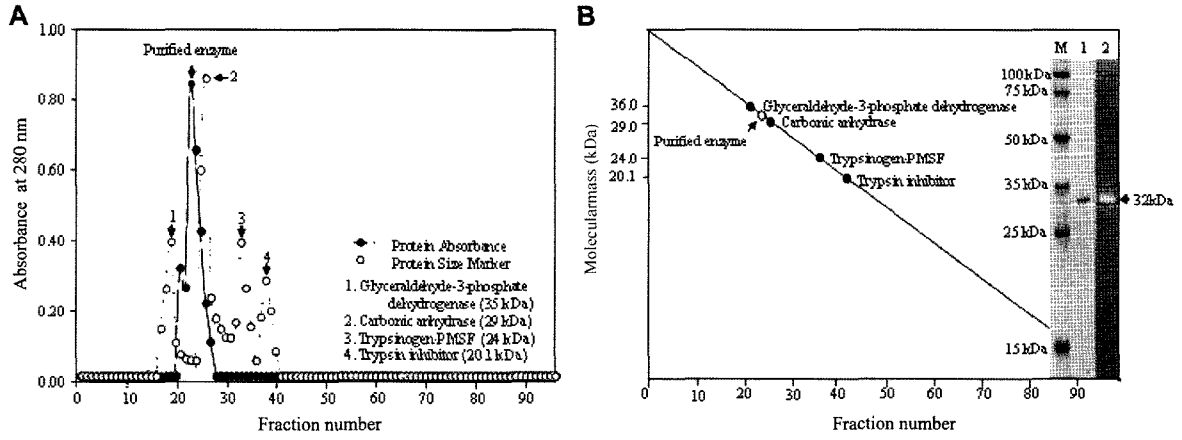

Fig. 2. Molecular mass determination of fibriolytic metalloprotease using size-exclusion on HiLoad 16/60 superdex 75 column (**A**) and semilogarithmic plot and fibrin-zymography (**B**).
A. The standard marker was eluted through a HiLoad 16/60 superdex 75 column equilibrated with 0.05 mM phosphate buffer containing 0.15 M NaCl, pH 7.4, at a flow rate of 1 ml/min. The elution profiles were monitored by spectrophotometry at 280 nm. **B**. Fibrin-zymography was carried out in 10.5% polyacrylamide gel containing 0.12% fibrin. After immersion of the gel plate in 10 mM Tris-HCl (pH 7.4) to grade the fibrin, the fibrinolytic region in the gel was revealed as a colorless band with a blue background, by Coomassie brilliant blue R-250.

PoFE	A	L	R	K	G	G	A	A	A	L	N	I	Y	S	V	G	F	T	S
AAU94648	A	L	R	K	G	G	A	A	A	L	N	I	Y	S	V	G	F	T	S
AAQ07436	S	L	R	K	G	G	A	A	A	L	N	V	Y	F	L	K	D	L	G

Fig. 3. Homology analysis of purified fibrinolytic metalloprotease amino acid sequence from mycelia of *P. ostreatus*.
The shadow indicates similar amino acid residues between the purified enzyme and other proteolytic enzymes. PoFE, the purified fibrinolytic metalloprotease from mycelia of *P. ostreatus*; AAU94648, metalloprotease from the fruiting body of *P. ostreatus*; AAQ07436, metalloprotease 1 precursor from *Coccidioides posasasii*.

(GenBank Accession No. AAU94648). As shown in Fig. 3, the N-terminal amino acid sequences of purified PoFE had a high homology to PoMEP of *P. ostreatus* fruiting bodies (AAU94648).

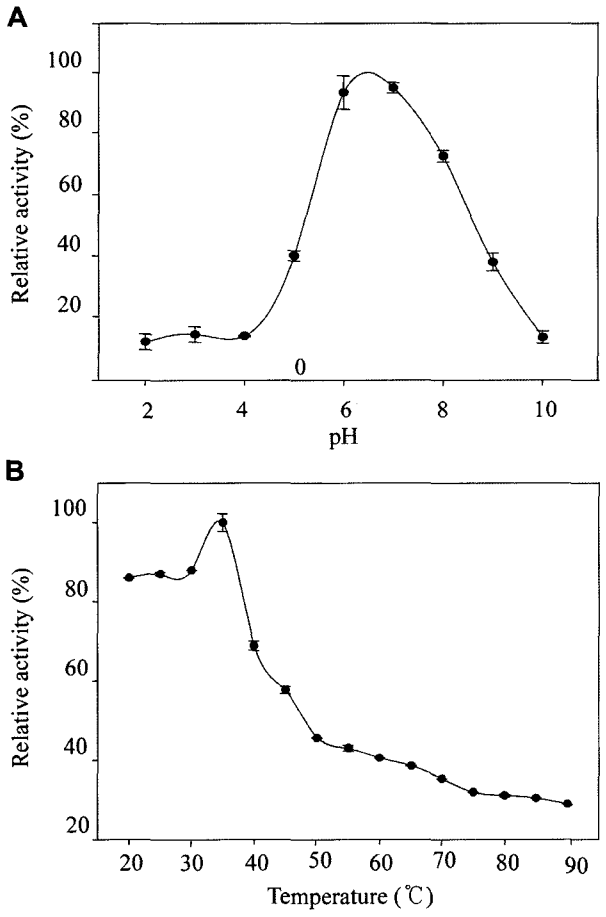

Fig. 4. Effect of pH (**A**) and temperature (**B**) on the activity of the fibrinolytic metalloprotease from mycelia of *P. ostreatus*.
A. Enzyme activity was assayed in the pH range of 2–10. The 0.5 M glycine-HCl (pH 2.0–3.0), 5 M acetate (pH 4.0–5.0), 0.5 M Tris-HCl (pH 6.0–8.0), and 0.5 M glycine-NaOH (pH 9.0–10.0) buffers were used with 0.1% azocasein. Enzyme activity was measured by incubation for 1 h at various pH values and 37°C. **B**. Effects of temperature on the activity of the fibrinolytic metalloprotease purified from mycelia of *P. ostreatus*. The purified enzyme was incubated at temperatures from 20 to 80°C. Enzyme activity was measured by azocasein assay at 366 nm.

Table 2. Effect of protease inhibitors on the activity of the fibrinolytic metalloprotease purified from mycelia of *P. ostreatus*.

Protease inhibitors	Concentration (mM)	Relative activity (%)
Control	-	100
PMSF	1.0	78
TLCK	1.0	81
TPCK	1.0	74
EDTA	1.0	52
Aprotinin	1.0	88
Pepstatin A	1.0	88

Effect of pH and Temperature on Fibrinolytic Activity

The effect of pH on the activity of purified enzyme from *P. ostreatus* was determined using buffers at various pH. As shown Fig. 4A, this result indicated that purified enzyme from *P. ostreatus* was active over a wide pH range (2.0–10.0), and exhibited maximum activity at pH 6.5. The enzyme was very stable in a pH range of 6.0–7.0, at 37°C for 1 h, but above pH 7.0, enzyme stability was decreased. As shown in Fig. 4B, the effect of temperature on the activity revealed that the enzyme was active between 20 and 50°C. Optimum activity was found to occur at 35°C. However, when exposed for 1 h to a temperature of over 37°C, the activity of purified enzyme decreased dramatically.

Effect of Inhibitors and Metal Ions on the Fibrinolytic Activity

The effect of various inhibitors on fibrinolytic activity is summarized in Table 2. The purified fibrinolytic enzyme was inhibited by 1.0 mM EDTA, a well-known metalloprotease inhibitor. As shown in Table 3, the effects of various metal ions on enzyme activity were assessed by assaying residual enzyme activity after the incubation of the enzyme with 1.0 mM of metal ions for 1 h at 37°C. The enzyme activities were found to be enhanced by Ca^{2+}, Mg^{2+}, and Zn^{2+}, but were inhibited by the Co^{2+}, Cu^{2+}, and Fe^{2+}, ions.

Analysis of Fibrinolysis and Fibrinogenolysis

The hydrolysis of fibrin by purified enzyme was analyzed by SDS-PAGE. As shown in Fig. 5A, the purified enzyme

Table 3. Effect of metal ions on the activity of the fibrinolytic metalloprotease purified from mycelia of *P. ostreatus*.

Metal ions	Concentration (mM)	Relative activity (%)
Control	-	100
Ca^{2+}	1.0	118
Co^{2+}	1.0	96
Cu^{2+}	1.0	87
Fe^{2+}	1.0	91
Mn^{2+}	1.0	94
Mg^{2+}	1.0	122
Zn^{2+}	1.0	114

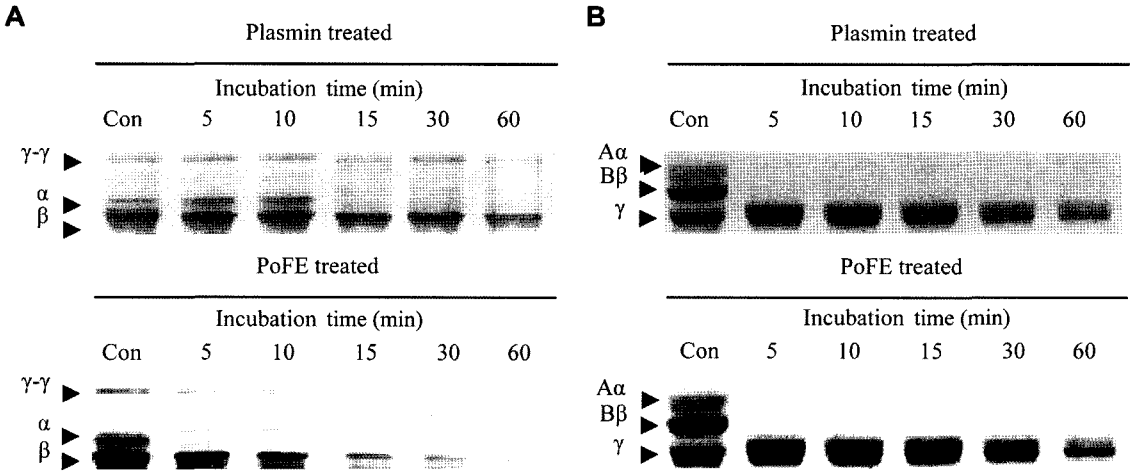

Fig. 5. Fibrinolysis (**A**) and fibrinogenolysis (**B**) patterns exhibited by fibrinolytic metalloprotease purified from mycelia of *P. ostreatus*.
Fibrin and fibrinogen were incubated with purified fibrinolytic metalloprotease for the various times indicated. Plasmin was used as the positive control.

rapidly hydrolyzed the α-chain, followed by the β-chain. The purified enzyme also hydrolyzed the γ-γ chains, but was a more slow digest than the α-chain and β-chains. Moreover, purified enzyme had fibrinogenolytic activity, and the degradation pattern of fibrinogen by purified enzyme was analyzed by SDS-PAGE (Fig. 5B). As shown in Fig. 5B, purified enzyme rapidly hydrolyzed Aα, and Bβ chains, whereas the γ-chain was hydrolyzed after 1 h of incubation time.

Amidolytic Activity of PoFE

The amidolytic activity of purified fibrinolytic enzyme was assessed with several chromogenic substrates. As shown in Fig. 6, the fibrinolytic enzyme exhibited a higher degree of specificity for the substrate S-2586 for chymotrypsin (MeO-Suc-Arg-Pro-Tyr-*p*NA·HCl). Therefore, purified enzyme from *P. ostreatus* was considered to be a chymotrypsin-like metalloprotease.

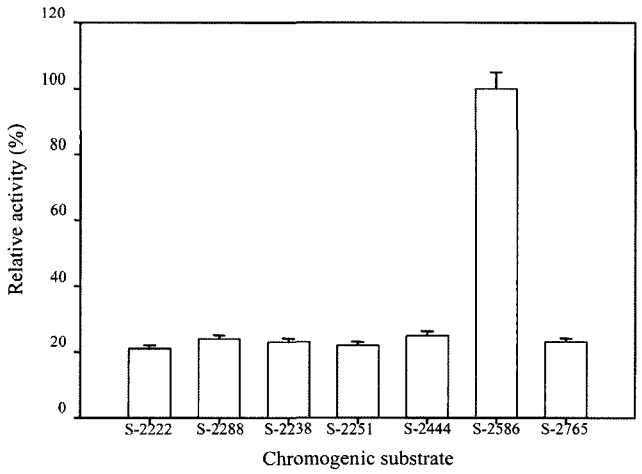

Fig. 6. Amidolytic activity on several chromogenic substrates.
Amidolytic activities were measured spectrophotometrically using chromogenic substrates.

cDNA Cloning and Sequence Analysis

After isolation of total RNA, the target gene was amplified from the cDNA by PCR. Two fragments of the PCR product of the PoFE protein-encoding gene from *P. ostreatus* mycelia (Fig. 7A) were cloned into pGEM-T easy vector (Figs. 7B and 7C) and were designated PoFE-867 (867 nt) and PoFE-1189 (1,189 nt), respectively. The resulting DNA sequence was analyzed with Sequence Database searches using BLAST sequence comparison algorithms at NCBI (http://www.ncbi.nlm.nih.gov/BLAST/ http://www.ncbi.nlm. nih.gov/BLAST/). The nucleotide sequence and the deduced amino acid sequence of the target gene are presented in Fig. 8 and 9. The nucleotide sequence revealed an open reading frame (ORF) of 867 bp encoding 288 amino acid residues. DNA sequence similarity analysis against GenBank by using the Blast program showed that the PoFE cDNA has 97% identity with PoMEP (GenBank Accession No. AY640032) and has a significant identity with metalloprotease from *Metarhizium anisopliae* var. *anisopliae* (CAB63909). The aminoacid sequence of PoFE purified from *P. ostreatus* mycelium has an extended zinc-binding consensus sequence (HEXXHXUGUXH), and a so-called Met-turn sequence (Fig. 9B), which is typical for the metzincin family of metalloproteases, suggesting that it is a metzincin metalloprotease. In addition, PoFE metalloprotease showed a significant homology with other fibrinolytic mushroom metalloproteases such as *Armillaria mellea* (CAB42792) and *Grifola frondosa* (BAB82381).

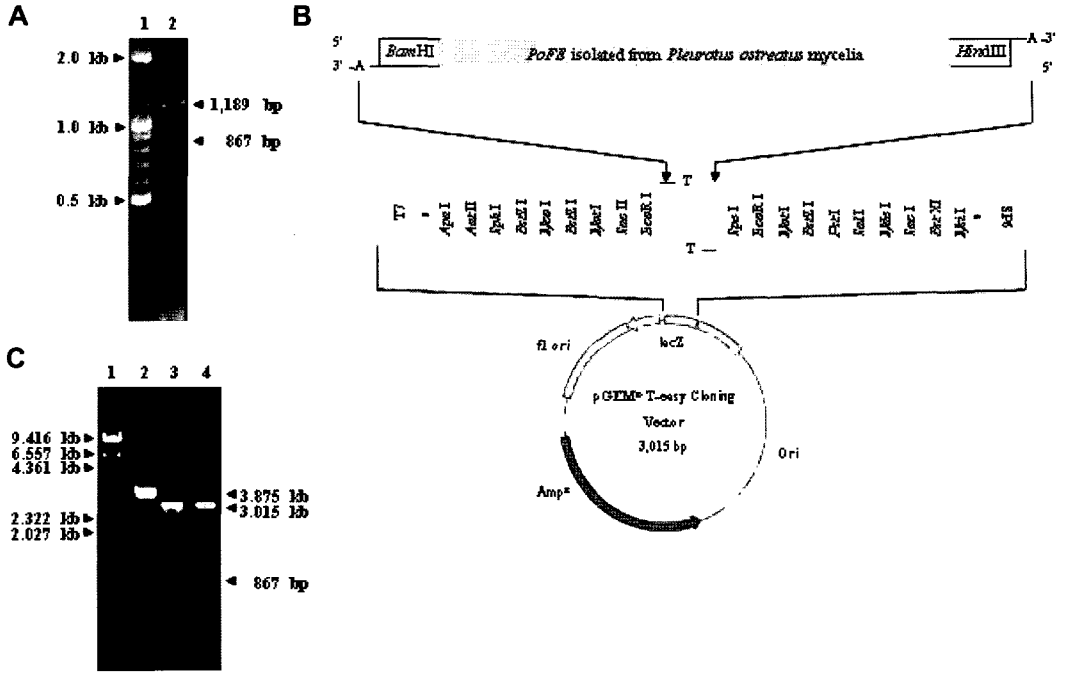

Fig. 7. *PoFE* isolation (**A**) from *P. ostreatus* mycelia, the cloning scheme (**B**), and cloning into pGEM T-easy cloning vector (**C**).
A. RT-PCR was performed using specific PCR primers that were designed for the conserved sequences in the metalloprotease-encoding gene from *P. ostreatus* fruit body (NCBI Accession No. AY 640032.1). Lane 1, 100-bp ladder DNA marker; lane 2, PCR products. **C.** Recombinant DNA of *PoFE* into pGEM T-easy vector. Lane 1, λ-HindIII DNA marker; lane 2, Restriction enzyme-digested recombinant DNA treated by HindIII; lane 3, Restriction enzyme-digested recombinant DNA treated by EcoRI; lane 4, Restriction enzyme-digested pGEM T-easy vector treated by PstI. It was resolved by electrophoresis on a 0.8% agarose gel and then visualized by EtBr staining of the gel.

Discussion

In this report we have described the purification, characterization and cloning of a fibrinolytic enzyme from the mycelia of edible oyster mushroom, *P. ostreatus*. Enzyme was purified 876-fold for a total yield of 6.5% (Table 1). The molecular mass of purified enzyme was calculated as 32 kDa by gel filtration, SDS-PAGE, and fibrin-zymography, which was similar to that determined for the metalloendopeptidases from *A. mellea* (32 kDa) [35] but higher than the metalloendopeptidases from the fruiting body of *P. ostreatus* (19 kDa) [17, 38] and *G. frondosa* (20 kDa) [38]. The first 19 amino acid residues of the N-terminal sequence of purified enzyme were ALRKGG AAALNIYSVGFTS, which is similar to the metalloprotease purified from the fruiting body of *P. ostreatus* (GenBank Accession No. AAU94648.1). In general, temperatures closest to the physiological temperature of the source from which the enzymes were obtained allow for optimal activity [52]. The optimum temperature of purified enzyme was 35°C, but when the enzyme was exposed to temperatures of over 37°C, the fibrinolytic activity of the enzyme degenerated abruptly (Fig. 4B). The possibility could be denaturation of protein above 37°C, causing a decreased in activity. As the temperature increased above 37°C, the enzyme was rendered inactive. It could be possible, because as the temperature rises, this causes a change in the secondary and tertiary levels of protein structure, and the active site is altered in its conformation beyond its ability to accommodate the substrate molecules it was intended to catalyze. The optimum pH of purified enzyme was 6.5, which is comparable to those of FP I and FP II from *Pleurotus sajor-caju* [41], MEF from the egg cases of *Tenodera sinensis* [18, 19], PoMEP from the fruiting body of *P. ostreatus* [38], and AmMEP from *Armillaria mellea* [35].

Considering the fibrinolysis pattern, it was found that the purified enzyme rapidly hydrolyzed the α-chain, followed by the β-chain. The purified enzyme also hydrolyzed the γ-γ chains, but more slowly. Moreover, fibrinogenolysis pattern of purified enzyme revealed that it rapidly hydrolyzed Aα and Bβ-chains, while γ-γ chain was hydrolyzed after 1 h of incubation time. Since purified enzyme directly hydrolyzes the plasminogen-free fibrin plate, this enzyme does not exert its fibrinolytic activity *via* plasminogen activation. This enzyme acts directly on the fibrin clot just like plasmin, but it is expected to have a different activation mechanism since it is a metalloprotease.

The activity of purified enzyme (PoMEP) was enhanced by the addition of Ca^{2+}, Mg^{2+}, and Zn^{2+} ions, but was inhibited by the Co^{2+}, Cu^{2+}, and Fe^{2+} ions. PoMEP belongs to a family of zinc-dependent metalloproteases. Our data regarding the effect of these metal ions on the activity of

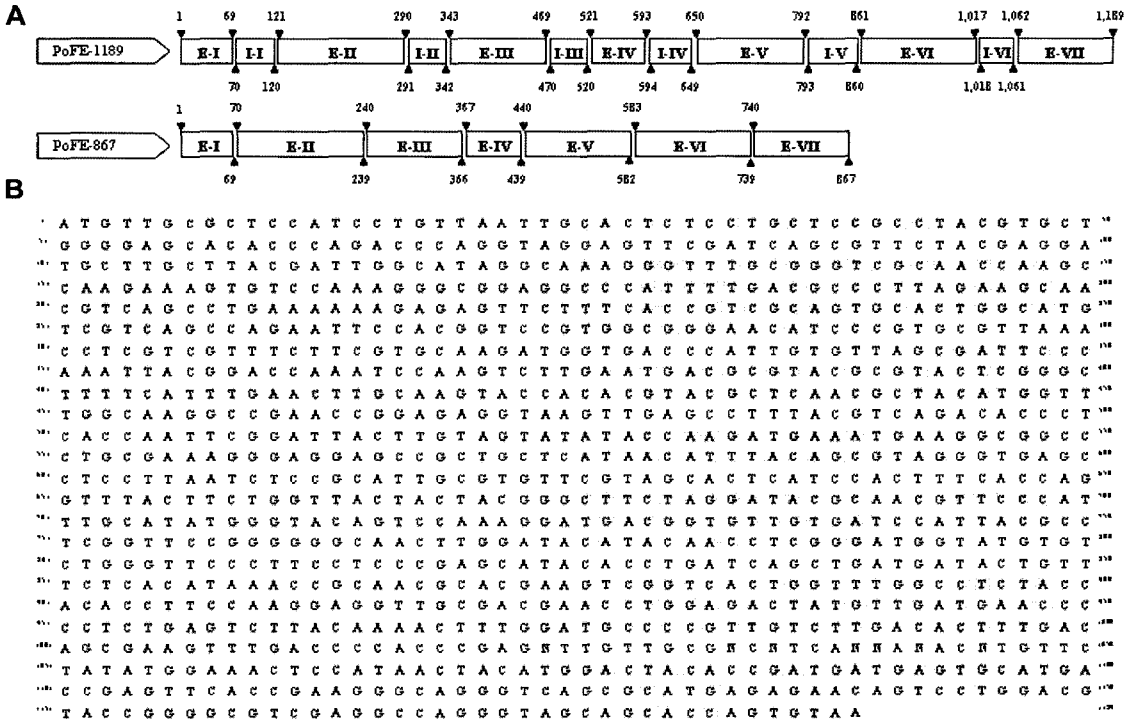

Fig. 8. DNA sequence analysis of the PoFE PCR product.
A. DNA structure comparison of *PoFE*-1189 and *PoFE*-867. **B.** Nucleotide sequence of the *PoFE* (1,189 bp) gene encoding the precursor form of the fibrinolytic enzyme of *P. ostreatus* mycelia. Shade indicates the PoFE-coding region.

the enzyme were consistent with the view that the fibrinolytic enzymes belonging to metalloproteases require divalent metal ions for their activities [39] (e.g., Zn^{2+} for *jeot-gal* [26], Ca^{2+} and Mg^{2+} for AMMP [35]), so their activities can be inhibited by chelating agents such as EDTA. Ca^{2+}, Mg^{2+}, and Zn^{2+} increased the protease activity; this is possible because of the activation by the metal ions. In addition, amino-acid sequence analysis suggested that zinc is required for the activity of this enzyme. Stricklin and Hibbs [42] reported that Ca^{2+} was required both for the activity and structural stability of metalloproteases. Therefore, it was not surprising that the activity of enzyme was increased when $CaCl_2$ was present. Furthermore, Co^{2+}, Cu^{2+}, and Fe^{2+} decreased the protease activity, because these metal ions could induce conformational change of the protease, which could account for the low activity. In general, the cations perform a specific role in the modulation of enzyme activity, whereas anions play a more general role in the modulation of the activity of enzymes [51]. It could be hypothesized that copper ion intrudes on the enzyme-catalyzed system by binding to carboxyl groups of the enzyme. In addition, the ferrous and cobalt ions could be unstable and oxidized, resulting in an inactive enzyme.

It was found that PoMEP exhibited a higher degree of specificity for the substrate S-2586 for chymotrypsin, indicating that the enzyme is a chymotrypsin-like metalloprotease. Interestingly, the fibrinolytic enzyme isolated from the *P. ostreatus* fruiting bodies was a lysine-specific metalloendoprotease [38], whereas the enzyme isolated from the *P. ostreatus* mycelia was a chymotrypsin-like metalloendoprotease.

The isolated fibrinolytic enzyme gene, *PoFE*, from the *P. ostreatus* mycelium was cloned and its nucleotide sequence was determined. Although we designed primers based on the nucleotides sequence of PoMEP isolated from the *P. ostreatus* fruit body, there were differences between the sequence of our PCR product and PoMEP, indicating the differentiation of gene expression in fruit bodies and mycelia and is inconsistence with the previous report by Lee *et al.* [34] and Sunagawa and Magae [46]. The cDNA of cloned PoFE is 867 nucleotides long and consists of an open reading frame encoding 288 amino acid residues (Fig 8). DNA sequence similarty comparison against GenBank by using the Blast program showed that the PoFE-867 cDNA has 97% identity with PoMEP [24] and has a significant identity with the metalloprotease from *Metarhizium anisopliae* var. *anisopliae* [15]. It has been suggested that PoMEP metalloprotease plays an important role in mushroom fruiting [24]. Interestingly, unlike PoMEP from fruit body, PoFE metalloprotease showed a significant homology with other prereported fibrinolytic mushroom metalloproteases of *Armillaria mellea* and *Grifola frondosa* [20, 38]. His-Glu-X-X-His or HExxH, where X is any non-conserved amino acid, is the consensus

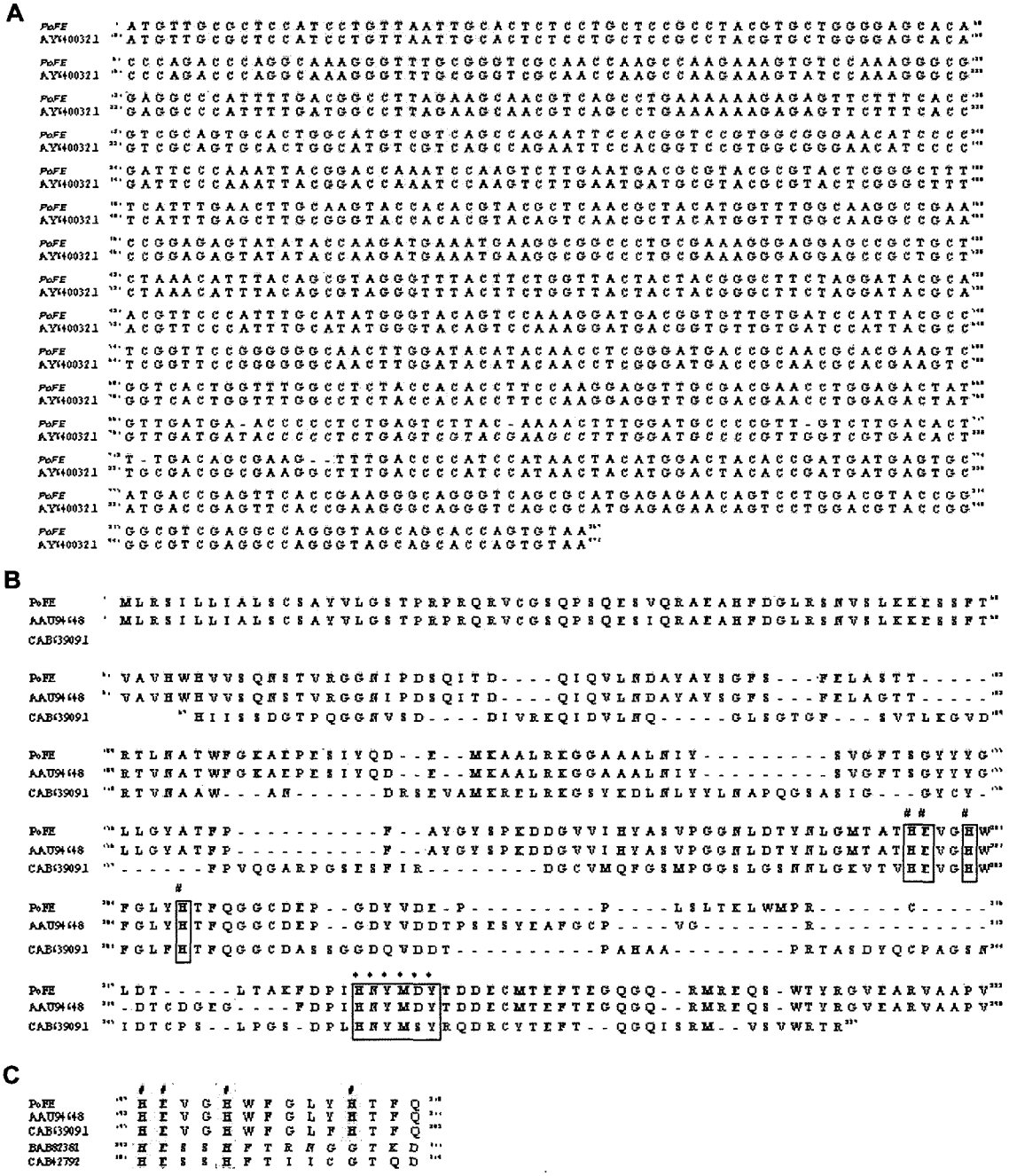

Fig. 9. Alignment and domain analysis of the *PoFE* DNA sequence and its deduced amino acid sequence.
A. Alignment of *PoFE* DNA sequence with AY640032.1 (isolated from *P. ostreatus* fruit body). **B.** Alignment and domain analysis of PoFE deduced amino acid sequence with AAU94648 (purified from *P. ostreatus* fruit body) and CAB63909.1 (purified from *Metarhizium anisopliae* var. *anisopliae*). # indicates the Zn-binding domain. * indicates the Met-turn homology region. **C.** Alignment of the Zn-binding domain (HEXXH) of various mushroom-originated metalloproteases, AAU94648 (originated from *P. ostreatus* fruit body), CA63909.1 (originated from *Metarhizium anisopliae* var. *anisopliae*), BAB82381 (originated from *Glifora frondosa*), and CAB42792 (originated from *Armillaria mellea*).

sequence for the active site in some zinc-dependent endopeptidases and aminopeptidases [47]. The amino acid sequence of PoFE purified from the *P. ostreatus* mycelium has an extended zinc-binding consensus sequence (HEXXHXUGUXH), and a so-called Met-turn sequence, which are typical for the metzincin family of metalloproteases, suggesting that it is a metzincin metalloprotease (Fig. 9). Bodea *et al.* [4] 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 [40]. The PoMTP was proposed to be grouped together with a series of putative fungal orthologs into the separate metzincin family "eucolycins" and has an extended zinc-binding consensus sequence (HExxHxxGxxH) [24], which is typical for the metzincin metalloprotease family. Besides PoMEP, a noticeable similarity was also found with other metzincin prototypes, pappalysins, astacins, serralysins, adamalysins/ADAMs, and matrix metalloproteinases (data not shown).

The overall results suggest that the fibrinolytic enzyme obtained from the edible oyster mushroom *P. ostreatus* shows a high degree of specificity toward fibrin. Hence, it could be useful in thrombolytic therapy as it is a directly acting thrombolytic agent, since direct-acting fibrinolytic enzymes are more effective and are unique thrombolytic drugs distinct from the plasminogen activators [29]. In addition, it will provide an adjunct to the costly fibrinolytic enzymes that are currently used in managing heart disease, since large quantities of enzyme can be conveniently and efficiently produced. However, clinical results are necessary for the thrombolytic application, but *P. ostreatus* may become a new source for thrombolytic agents.

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