Biochemical and molecular characterization of fibrinolytic enzyme purified from *Pleurotus ostreatus*

(Received: 03. 05. 2016; Accepted: 25. 05.2016)

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ABSTRACT

The crude fibrinolytic enzyme was purified to full homogeneity using fractional precipitation by ammonium sulphate, DEAE cellulose chromatography and gel filtration on Sephadex-G100. An over all of 69%-fold purification with 2.5 recovery was obtained. The apparent molecular mass of the purified enzyme was estimated to be 44 kDa using SDS-PAGE. The optimum pH and temperature of the purified enzyme were 6 and 35˚C, respectively. The fibrinolytic enzyme was completely inhibited by Hg^{2+} and partially inhibited by Cu^{2+}, Ni^{2+} and Al^{3+}. Its activity strongly enhanced by Zn^{2+}, Mg^{2+}, Ca^{2+}, K^{+} and Fe^{2+} in descending order. EDTA and EGTA inhibited the enzyme activity suggesting that it is a metalloprotease. The enzyme was also inhibited by the serine inhibitors PMSF and aprotinin. The pure enzyme showed strong specificity to fibrin as substrate in vitro. It was also specific to gelatin and fibrinogen but not specific to casein, elastin and egg albumin. The amidolytic activity toward synthetic substrates showed high specificity to the synthetic peptide N-Succinyl-Ala-Ala-Pro-Phe-pNA suggesting that it is a chymotrypsin-like protease. The PoFR-cDNA encoding gene was cloned in E. coli (α-DH5) and its nucleotide sequence was determined (GenBank accession no. AB551656). The PoFR-cDNA was found to consist of 845 bp in an Open Reading Frame (ORF) encoding 281 amino acids. The sequence showed high degree of homology with the fibrinolytic enzyme gene from P. ostreatus fruiting bodies (AY640032.1). In vivo assay using thrombus induced mice showed that the enzyme exerted thrombolysis in the mice blood marked by decrease in hematocrit percentage and prolonged prothrombin time (PT) and thrombin time (TT).

***Key words**: Pleurotus ostreatus, Fibrinolytic enzymes, Thrombosis, Thrombolysis.*

INTRODUCTION

Fibrin is the primary protein component of a blood clot, which is formed from fibrinogen by thrombin (Voet and Voet, 1990). Fibrin causes thrombosis, leading to myocardial infarction and other cardiovascular diseases in the blood vessels. The insoluble fibrin fiber is hydrolyzed by plasmin, which is generated from plasminogen by plasminogen activators, such as tissue plasminogen activator, vascular plasminogen activator, blood plasminogen activator, urokinase, Hageman factor, and streptokinase-plasminogen complex (Collens and Lijnen, 1991). The hydrolysis of fibrin is known as fibrinolysis. Fibrin clot formation and fibrinolysis are normally well balanced

in the biological system. However, in the unbalanced state, the clots are not lysed, and therefore thrombosis occurs as mentioned by Harlan and Harker (1981). 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 search for other fibrinolytic enzymes from various sources continue. Over the last decade, potent fibrinolytic enzymes have been discovered from a variety of sources, such as earthworms (Mihara *et al.*, 1991 and Wang *et al.*, 2006), snake venoms (Jia *et al.*, 2003 and De-Simone *et al.*, 2005), insects (Ahn *et al.*, 2003), food-grade microorganisms (Ang *et al.*, 2005), marine creatures (Sumi *et al.*, 1992), herbal medicines (Harlan and Harker, 1981) and fermented food products like Japanese natto (Sumi *et al.*, 1992, Choi *et al.*, 2005 and Stricklin and Hibbs, 1988), Korean chungkook-jang (Kim *et al.*, 2006).

In recent years, mushrooms have become an attractive source of various physiologically active compounds (Wang *et al.*, 2005 and Woessner, 1991). 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 (Collens and Lijnen, 1991). They constitute an important source of thrombolytic agents. Many fibrinolytic enzymes were identified in the fruiting bodies of different medicinal mushrooms, such as *Armillaria mellea* metalloprotease, (Healy *et al.*, 1999 and Kim and Kim 1999). *Grifola frondosa* aminopeptidase (Nonaka *et al.*, 1997) and *Pleurotus ostreatus* metalloprotease (Choi and Shin, 1998 and Joh *et al.*, 2004). Indeed, the presence of fibrinolytic enzymes in the fruiting bodies of some mushrooms has been identified, although the presence of these enzymes in their 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.

The present study aimed to the purification, biochemical and molecular characterization of the fibrinolytic enzyme. *In vivo* assay of thrombolysis activity using thrombus-induced mice model was demonstrated with the aid of some markers.

MATERIALS AND METHODS

Organism and culture conditions

The white-rot fungus *Pleurotus ostreatus* (Jacq.) Que'let NRRL0366 (oyster mushroom) was kindly provided by Scientific City for Scientific Research and Technology, Alexandria, Egypt. The fungus was maintained on PDA medium described by Prasad *et al*. (2005).

Fibrinolytic enzyme assay

Fibrinolytic enzyme activity in the culture filtrate was measured spectrophotometrically as described by Datta *et al*. (1995). Ten µg of human fibrinogen solution (prepared in 10 mM Tris-HCl buffer pH 7.4 containing 0.15 M NaCl) were added to human thrombin (0.1 NIH unit), and allowed to stand for 1 h at room temperature. The formed clots (fibrin) were mixed with 1 ml of filtrate (as a source of crude fibrinolytic enzyme) and incubated at 37°C for 1 h. The amount of solubilized peptides was measured

per one ml reaction mixture using procedure of Bradford (1976). One unit of enzyme activity was expressed as the amount of enzyme releasing 1µmol of soluble peptides per minute

Proteolytic enzyme assay

Proteolytic activity was determined according to Shen *et al.* (2007) by measuring the release of acid-soluble material from azocasein by absorbance at 366 nm. One unit of protease activity was defined as the amount required producing enough acid-soluble material from azocasein to yield an absorbance of 0.1 at 366 nm after 1 h of incubation at 37°C.

Purification of fibrinolytic enzyme in *P. ostreatus*

The culture filtrate (1.5 liters) was fractionated by precipitation with ammonium sulfate $(NH_4)_2SO_4$ with up to 80% saturation at 4°C. The precipitate was dissolved in least amount of 20 mM Tris-HCl buffer (pH 7.4) and desalted by dialysis over night against the same buffer, kim et al., 2008. The previous partially purified enzyme extract was applied to DEAE-cellulose column (3.5 × 10 cm) (Pharmacia) equilibrated with 20 mM Tris-HCl buffer (pH 7.4). Proteins were eluted with linear gradient of NaCl from 0.0-0.7 M. The flow rate was 1.0 ml min^{-1} and Fractions of 3 ml were collected; protein content and fibrinolytic enzyme activity were assayed in each fraction. Fractions containing fibrinolytic activity from DEAE-cellulose column were pooled, concentrated by lyophilization, redissolved in 10 mM sodium-phosphate buffer pH 6.8 and applied to Sephadex G-100 column (Pharmacia- USA). Elution was carried out by the same buffer and fractions collected and assayed for fibrinolytic activity and protein content.

Molecular and Biochemical character-rization

The molecular weight of the enzyme was determined by SDS-PAGE according to the method described by Laemmli (1970). After electrophoresis. The gels were stained with silver nitrate and the molecular mass markers used were low molecular weight standards (Sigma- Germany). The effect of pH, temperature, protease inhibitors, metal ions and substrate concentration as well as specificity on fibrinolytic enzyme activity were studied.

Amplification of PoFR gene by RT-PCR

Isolation of RNA was carried out according to the method outlined by Huang *et al.* (2007) using Biozol reagent (BioFlux, Tokyo, Japan), and RNA was purified using tri- reagent RNA kit (Sigma). cDNA synthesis was carried out by using1st strand cDNA kit (BIO BASIC INC.) according to the manufacturer instructions, in a reaction mixture (20 µl final volume). Specific products were amplified by PCR, (using *T-GRADIENT* thermal cycler from Biometra) in 25 µl volume containing 2.5 µl of cDNA; 25 pmol of each primer (forward primer (Pofib1) AAT GGATCCATGTTGCGCTCCATCCTG and reverse (Pofib2) AATAAGCTT GGCTTCGTACGACTCAGAGG), 10 mM of each dNTP; 1 U of Taq DNA polymerase; 10 mM Tris-HCl, pH 9.0; 50 mM KCl; 1 mM $MgCl_2$. Conditions for PCR were denaturation at 94 ºC for 3 min followed by 35 cycles of 1 min at 94 ºC; 2 min at 60 ºC and 1 min at 72 ºC with a final extension step at 72º C for 7 min.

Cloning of PoFR into PGEM-T-Easy vector and sequencing

The generated DNA fragments of fibrinolytic gene obtained after PCR

amplification were purified using QiA quick gel extraction kit (Qiagen) according to manufacturer's instructions. The products were ligated into PGEM- T- Easy vector (system 1) from Promega (Madison,WI, USA), and transformed into competent DH5α- *E. coli* cells (Promega) with subsequent ampicillin selection following manufacturer's instructions. The PGEM- T-Easy cloning kit offered a rapid and efficient cloning method for PCR products. 2 µl of ligation reactions were added and mixed gently by tapping and incubated on ice for 30 min. Cells were heat shocked for 45 sec. at 42 ºC in a water bath incubator to increase the transformation efficiency. The tubes were then placed on ice for 1 min to cool down then 900 µl of LB medium were added to each tube and shaked gently at 37 ºC for 2 h for cell recovery. 100 µl transformation mix were plated onto Luria-Bertani (LB) plates containing ampicillin 50 mg/ml,100 mM IPTG and 50 µg/ml X-gal and incubated at 37ºC overnight for selecting the transformed cells. QiA prep® Miniprep kit (Qiagen) was used to isolate pure super-coiled plasmid DNA with high yields (15 µg) according to manufacturer's instructions. Validation of cloning took place by PCR to select the transformed colonies with recombinant PGEM-T Easy vector by using specific (Pofib1and Pofib2 primers) and universal primers (M13 forward and reverse). Partial nucleotide sequences of *PoFR* gene were obtained by the sequencing of one strand on an Applied Biosystems 310 genetic analyzer (Applied Biosystems, ABI) using sequencing ready reaction Mix according to manufacturer's instruction (Applied Biosystem) at Gene Analysis Unit, VACSERA, and Cairo, Egypt.

In vivo* assay of thrombolysis activity using purified fibrinolytic enzyme in *P. ostreatus

Male and female ICR mice animals (4 to 6 week old with 20 to 25 g weight) were obtained from National Cancer Institute, Cairo University, Egypt. Two groups of five animals each were randomly allocated. Thrombin obtained from Sigma company was prepared and injected intravenously in foot of each mouse to initiate leg clot formation. After 1 h of intravenous injection (time required to allow clot to be formed) blood samples were collected from foot (control group). The mice groups were then injected with the purified enzyme samples of *P. ostreatus* for 3 days and another blood sample was collected from foot of each mouse in tubes containing anticoagulant. The two blood samples in addition to samples collected from untreated healthy mice group were analyzed for thrombin time (TT), prothrombin time (PT) and hematocrit percentage as described by Yuk *et al.* (2000).

RESULTS AND DISCUSSION

Enzyme Purification and Molecular Mass Determination

The partially purified enzyme obtained by fractional precipitation with ammonium sulphate recorded 57.3% recovery with 1.8-fold purification. Three separate peaks of fibrinolytic activities were observed named FA, FB and FC by using DEAE-cellulose (Fig. 1). The composite sample containing the three active peaks was further applied onto Sephadex G-100 chromatography where one main peak appeared showing high fibrinolytic activity (Fig. 2). An overall of 2.5-fold purification with 69.1% recovery were attained (Table 1). The apparent molecular mass from the relative mobility by the marker proteins on SDS-PAGE was estimated to be 44 kDa (Fig. 3).

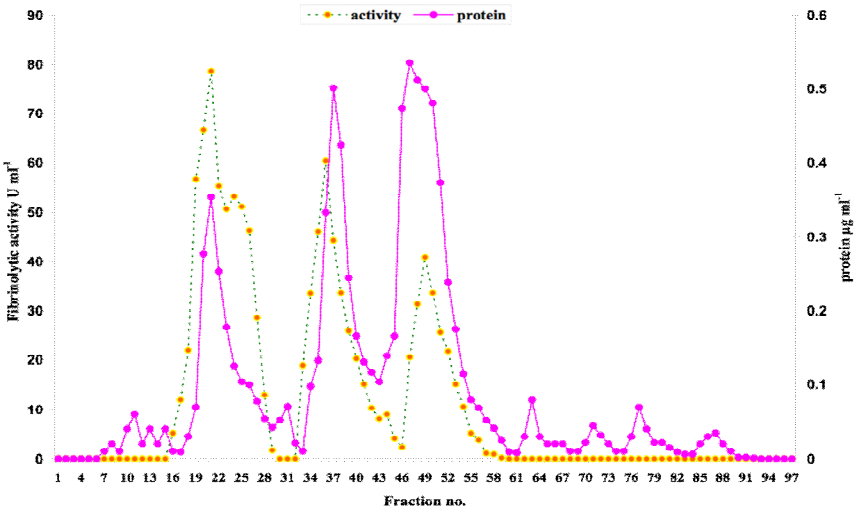

Fig. (1): Typical elution profile for the behavior of P. ostreatus extracellular fibrinolytic enzyme on DEAE-cellulos.

Table (1): Purification scheme of fibrinolytic enzyme in Pleurotus ostreatus .

Purification step		Total protein (µg ml^{-1})	Fibrinolytic activity (U ml^{-1})	Specific activity (7=U µg^{-1})	Purification fold	Recovery (%)
Crude filtrate		8.9	28.3	3.2	1	100
$(NH_4)_2SO_4$(80%)		5.1	30.3	5.9	1.8	57.3
DEAE-cellulose	**FA**	0.19	30.1	158.4	49.5	2.1
	FB	0.22	20.2	91.8	28.7	2.5
	FC	0.33	12.8	38.8	12.1	3.7
Sephadex G-100		0.22	48.7	221.4	69.1	2.5

FA= The 1st peak (fractions 16-29); FB= The 2nd peak (fractions 33-44); FC= The 3rd peak (fractions 46-57).

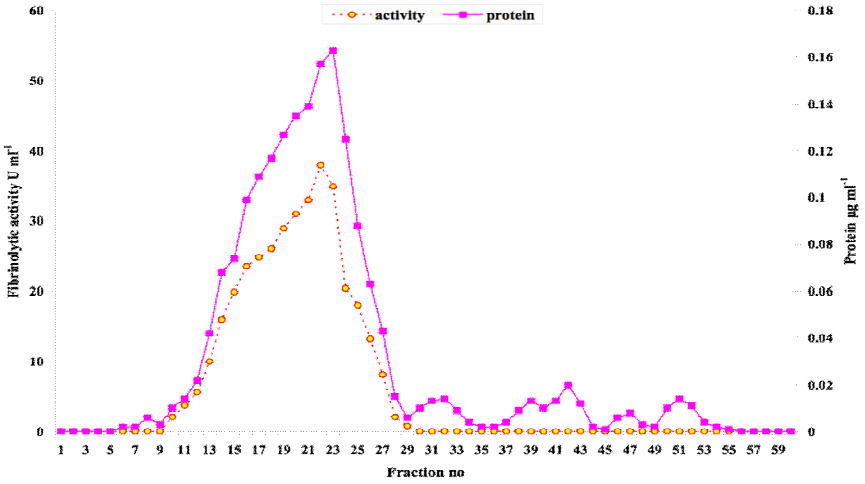

Fig. (2): Typical elution profile for the behavior of P. ostreatus extracellular fibrinolytic enzyme on Sephadex G-10.

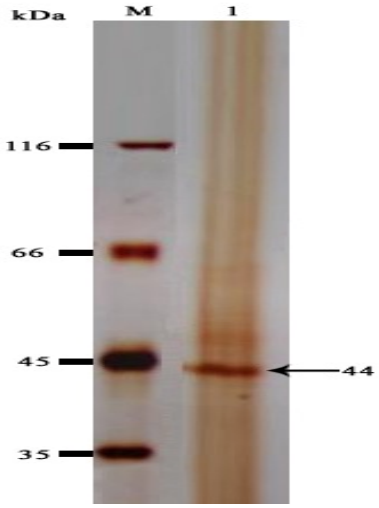

Fig. (3): SDS-PAGE for purified fibrinolytic enzyme from Pleurotus ostreatus; lane (M), Marker, protein standard including (β-galactosidase 116 kDa, bovine serum albumin 66 kDa, ovoalbumin 45 kDa, lactate dehydrogenase 35 kDa) and lane (1) purified fibrinolytic enzyme.

The enzyme was assigned as (PoFR) and similar or relatively similar molecular masses were observed by Paik *et al*., 2004 who purified fibrinolytic protease from *Bacillus subtilis* KCK-7 with an apparent molecular mass of 44 kDa. Kim *et al*. (2008) purified and characterized a fibrinolytic enzyme from mycelia of the mushroom *Perenniporia fraxinea* with apparent molecular mass of 42 kDa. Higher molecular masses than PoFR were detected by Kim *et al*., 2006 who purified a fibrinolytic enzyme from the medicinal mushroom *Cordyceps militaris* with molecular mass of 52 kDa by SDS-PAGE. However, lower molecular masses than PoFR were detected in *P. ostreatus* by Shen *et al*. (2007) who purified fibrinolytic enzyme from mycelia (32 kDa) and fruiting bodies (19 kDa) of *P. ostreatus* using combination of chromatographies.

Effect of pH values

The optimum pH for the enzyme was observed at pH 6.0 as given in Fig. (4). This enzyme was highly active at pH range of 5.0-8.0 at 37ºC for 1 h but the enzyme activity declined rapidly below or above this range. Similarly, the optimum pH of *P. ostreatus* purified fibrinolytic enzyme was 6.5. The enzyme was stable in pH range of 6.0-7.0 but above 7.0 the enzyme stability decreased drastically, Shen *et al*. (2007). Maximum activity of purified fibrinolytic enzyme was detected at pH 6.0 in the mushroom *Cordyceps militaris*, Cui *et al.,* 2008. The optimum pH of PoFR is also similar to those of FFP2 from *Prenniporea fraxinea* (Kim *et al.,* 2008 and Lee *et al.,* 2005). PoMEP from fruiting body of *P. ostreatus*, (Nonaka *et al.,* 1997), AMMP from *Armillaria mellea*, (Lee *et al.,* 2005), FP1 and FP2 of *P. sajor-caju*, (Shin and Choi, 1999). The fibrinolytic enzymes from *B. subtilis* DC-33 showed stability over a wide range of pH 5-12 with maximum activity at pH 8.0, (Wang *et al.,* 2006).

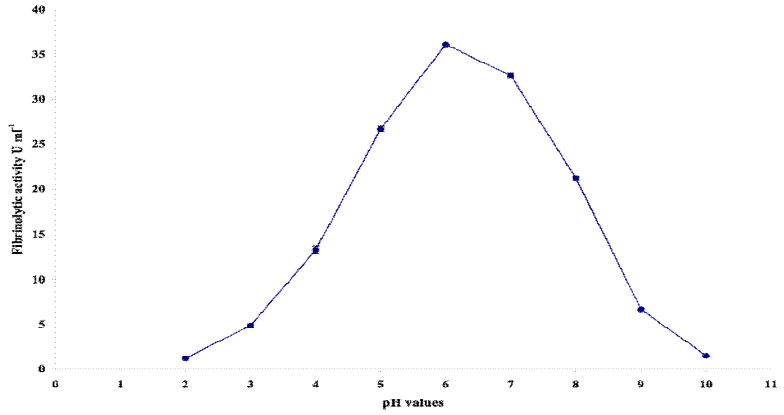

Fig. (4): Effect of different pH values on fibrinolytic enzyme activity from P. ostreatus.

Effect of temperature

The optimum temperature of the purified fibrinolytic enzyme from *P. ostreatus* was found to be 35ºC but when the enzyme was exposed to temperature over 37ºC the fibrinolytic activity degenerated abruptly as shown in Fig. (5). As the temperature increased above 45ºC the enzyme was rendered inactive. The fibrinolytic enzyme in *Cordyceps militaris* was active between 20-40ºC with an optimum activity at 37ºC. The activity decreased rapidly at temperature greater than 40ºC (Kim *et al.*, 2006). The optimum temperature of fibrinolytic enzyme purified from the mushroom *Perenniporia fraxinea* was found to be 35ºC. The enzyme become less active above 45ºC and completely inhibited above 55ºC, (Kim *et al.*, 2008). Lower temperature than that of PoFR was detected in other mushrooms by Cui *et al.*, 2008 who found that the optimum temperature of fibrinolytic enzyme purified from *Cordyceps militaris* mycelial filtrate was 25ºC. Lee *et al.* (2005) observed that the optimum temperature of fibrinolytic enzyme purified from the mushroom *Armillaria mellea* was 33ºC.

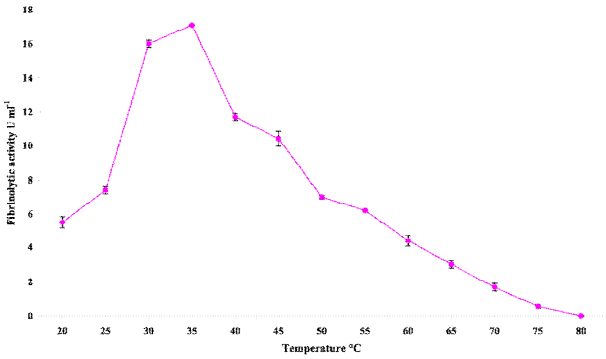

Fig. (5): Effect of different temperature values on fibrinolytic enzyme activity from P. ostreatus.

Effect of different protease inhibitors

Table 2 showed that EDTA and EGTA strongly inhibited the enzyme activity indicated that it's a metalloprotease. TPCK, DTT and mercaptoethanol exerted low effect on the fibrinolytic activity. The enzyme was inhibited by the serine protease inhibitors PMSF and aprotinin. The cysteine protease inhibitors TLCK and the aspartic protease inhibitor pepstatin A exerted low effect on the enzyme activity. Wang *et al*. (2006) reported that the fibrinolytic activity in *B. subtilis* DC33 completely inhibited by PMSF, DTT and pepstatin A which are well known inhibitors of serine proteases. Kim *et al*. (2008) indicated that the fibrinolytic activity of enzyme purified from the mushroom *Perenniporia fraxinea* was inhibited by EDTA and EGTA suggesting that it's a metalloprotease. No significant effects were exerted by PMSF, TLCK, aprotinin and pepstatin A.

Table (2): Effect of different protease inhibitors on fibrinolytic enzyme activity from P. ostreatus.

Inhibitors	Conc.	Activity (U ml^{-1})	% of change compared to control
Control	----	48.7±1.1	100
Aprotinin	0.2 mM	22.3±2.1*	54.2
Pepstatin A	0.2 mM	40.1±1.1*	17.6
EDTA	0.5 mM	18.2±2.1*	62.6
	1.0 mM	2.9±1.1	94.0
EGTA	0.5 mM	21.5±1.1*	55.8
	1.0 mM	5.2±0.1*	89.3
TLCK	1.0 mM	45.8±4.3*	5.9
TPCK	1.0 mM	16.1±3.2*	66.9
Dithiothreitol (DTT)	1.0 mM	32.5±2.1*	33.3
phenylmethylsulfonyl fluorid [PMSF]	1.0 mM	12.4±1.0*	74.5
2- mercaptoethanol	1.0 mM	35.2±3.2*	27.7

Effect of different metal ions on fibrinolytic enzyme activity

The effect of metal ions and protease inhibitors on the Fibrinolytic enzyme (PoFR) purified from *P. ostreatus* was investigated. The enzyme activity was completely inhibited by Hg^{2+} and partially inhibited by Ca^{2+}, Ni^{2+} and Al^{3+}. The fibrinolytic activity was strongly enhanced by Zn^{2+}, Mg^{2+}, Ca^{2+}, K^{+} and Fe^{2+} in decreasing order and moderately stimulated by Co^{2+}, Mn^{2+}, Fe^{3+} and Ba^{2+} (Table 3). It could be concluded that the fibrinolytic enzyme (PoFR) purified from *P. ostreatus* mycelium was enhanced by Zn^{2+} up to 140% appeared strongly as serine-metalloprotease requiring Zn^{2+} for its catalytic activity. This observation was also recorded in other microbial fibrinolytic enzymes. It has been reported that some fibrinolytic enzyme from various sources are serine and or metalloprotease requiring Zn^{2+}, Ca^{2+} or Mg^{2+} (Lee *et al.,* 2005 and Nonaka *et al.*, 1997). The fibrinolytic enzyme from *Rhizopus chinensis* 12 and *Streptomyces* sp. Y405 are both serine and metalloproteases (Liu *et al*.,2005). However, the fibrinolytic enzyme from *P. ostreatus* (Choi and Shin, 1998), *P. sajor-caju* (Fujita *et al.*,1995) is Zn^{2+} requiring metalloprotease.

Table (3): Effect of different metal ions at 1mM on fibrinolytic enzyme activity from P. ostreatus.

Metal ions	Activity (U ml^{-1})	% of change compared to control
Control	32.5±0.00	100
Mg^{2+}	73.7±2.1*	126.7
Co^{2+}	54.2±2.1*	66.8
Cu^{2+}	19.3±3.5*	40.6
Ca^{2+}	69.3±2.2*	113.2
Mn^{2+}	56.3±4.3*	73.2
Hg^{2+}	0.00±0.00	0.00
Zn^{2+}	78.0±2.1*	140
Fe^{2+}	66.1±1.1*	103.4
Fe^{3+}	52.0±4.3*	60
Ni^{2+}	22.7±2.1	32
Ba^{2+}	50.9±1.1*	56.6
K^{+}	68.2±1.1*	109.8
Al^{3+}	22.8±1.1*	29.8

Significant when P< 0.01

Substrate specificity, amidolytic activity, Km and Vmax determination

The enzyme showed highest specificity to the synthetic N-Succinyl-Ala-Ala-Pro-Phe-pNA (for subtilisin or chymotrypsin). High degree of specificity was shown also toward N-benzoyl-Phe-Val-Arg-pNA (for trypsin or thrombin). The enzyme (PoFR) was highly specific to fibrin, fibrinogen and gelatin but not specific to azocasein, elastine or albumin (Table 4). The Km and Vmax of the enzyme for N-Succinyl-Ala-Ala-Pro-Phe-pNA were determined to be 0.35 mM and 21.0 U ml-1, respectively. Similarly, when the fibrinolytic activity of *B. subtilis* DC-33 using fibrin as substrate (was taken as 100) the relative activity of the enzyme to fibrinogen, casein and serum albumin were 132, 18 and 12, respectively. It hydrolyzed N-Succinyl- Ala-Ala-Pro-Phe-pNA effectively with Km of 0.21 mM, Kcal/Km of $1.76x10^{5}$ $S^{-1}M^{-1}$, respectively. The enzyme also degraded D-Phe-Pipecolyl-Arg-pNA, aspartic substrate for thrombin with Km of 47.7 mM, Kcal/Km of 2.52 S^{-1} M^{-1} (Wang *et al.*, 2006). The fibrinolytic enzymes from *Rhizopus chinesis* showed high specificity for N-Succinyl-Ala-Ala-Pro-Phe-pNA and the Km value was 0.23 mM, Liu *et al.*, 2005. The fibrinolytic enzyme from mushroom *Fomitella fraxinea* showed a broad specificity for synthetic substrates and the km and Vmax values for N-Succinyl-Ala-Ala-Pro-Phe-pNA were 0.23 mM and 39.68 U ml^{-1}, (Lee *et al.*, 2005). The most sensitive substrate for fibrinolytic enzymes isolated from *B. lipoliquefacience* DC-4 was N-Succinyl-Ala-Ala-Pro-Phe-pNA for subtilisin or chymotrypsin, (Peng *et al.*, 2003).

Table (4): Effect of natural and synthetic substrates on Fibrinolytic enzyme activity.

Substrate	Conc.	Activity (U ml^{-1})	% of change compared to control
Fibrin	----	++++	----
Fibrinogen	----	++	----
Gelatin	----	++	----
Azocasien	----	_	----
Elastin	----	_	----
Albumin (egg)	----	_	----
N-Succinyl-Ala-Ala-Pro-Phe-p-nitroanilide	1.0 mM	75.8±2.1	100
N-benzyol-Phe-Val-Arg-p-nitroanilide	1.0 mM	47.4±1.1*	37.4
Gly-Phe-p-nitroanilide	1.0 mM	43.3±2.1*	43.0
Phe-Ala-p-nitroanilide	1.0 mM	33.6±3.2*	55.7
Gly-p-nitroanilide	1.0 mM	19.2±1.1*	74.7

The isolated fibrinolytic enzyme gene (*PoFR*-cDNA) from *P. ostreatus* mycelia has been cloned in *E. coli* (α-DH5). The nucleotide sequence of the cloned fibrinolytic gene revealed a single open reading frame (ORF) of 845 bp encoding 281 amino acids with start code ATG. The *PoFR*-cDNA gene exhibited high sequence homology (95%) with *P. ostreatus* metalloprotease (GenBank Accession No. AY640032.1). The calculated molecular mass was estimated to be 44 kDa and the theoretical isoelectric point (pI) was 4.74. The mycelia of *P. ostreatus* may thus represent a potential source of new therapeutic agents to treat thrombosis

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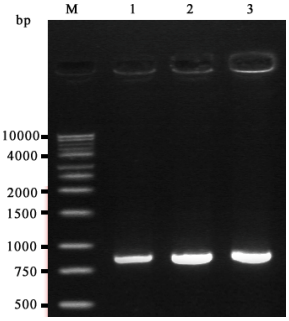

Fig. (6): PCR products of fibrinolytic enzyme gene (PoFR-cDNA). Lane M, DNA marker; Lanes 1, 2 and 3 PCR products (845bp).

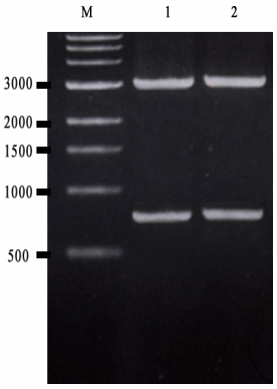

Fig. (7): Agarose gel electrophoresis of restriction digestion of fibrinolytic gene clones. Lane M, DNA ladder; Lane 1 and 2 digested gene clones G1 and G2 respectively; with Not1 restriction endonuclease.

Joh *et al.* (2004) isolated full length cDNA sequence (1140 bp) of PoMTP from *P. ostreatus* fruiting bodies which contained a 870 bp open reading frame encoding protein product of 290 amino acids in addition to a 99 bp of 5'-unsaturated sequence and a 171 bp of 3'-unsaturated sequence with a poly(A) tail. Shen *et al.* (2007) reported that the cDNA of cloned PoFE extracted from *P. ostreatus* mycelia is 867 nucleotides long and consist of an open reading frame encoding 288 amino acid residues (Figure 6 and 7). The deduced amino acid sequence and the first 18 signal amino acids showed high homology (90%) with PoMTP (Accession No. AAU94648.1) from *P. ostreatus* fruiting bodies. The zinc-binding domain was determined on our deduced sequence as "HEVGHFGLNH" and beneath it there was a methionine-turn "HFYMDY". This Met-turn sequence maintains the structure of zinc-binding site, which is typical for the metzincin family of metalloproteases, suggesting that PoFR is a metzincin metalloprotease. In addition, there were four cysteine residues, which form disulphide bridges (Fig. 8 and 9).

Several studies identified unique signatures within the amino acid sequences of the zinc metalloproteases and placing the enzymes into distinct family groups on the basis of sequence and more recently, structural similarities (Vallee, and Auld, 1990). Jiang and Bond (1992) compared the sequences around the HEXXH motif to classify zinc metalloproteases into five distinct families, thermolysin, astacin, serratia, matrixin and reprolysin, metalloproteases. The last four families have an extended zinc binding site "HEXXHXXGXXH" where the third histidine acts as the third ligand instead of glutamic acid in thermolysin. Astacins metalloproteases and snack venom fibrinolytic enzyme exhibited identical zinc-binding environments (His-Glu-X-X-His-X-X-Gly-X-X-His) and this was a consensus sequence in metalloprotease (Poindexter *et al.*, 1999).

All PoMTP-type metalloprotease belonging to the eucolysins subfamily reserves well four cysteine residues with the almost residues spacing in zinc-binding and Met-turn sequences ($HEXXHXXGXXHT^{12}$-C^{17}-C^{37}-C^{44}-$NYMDY^{58}$-C^{63}-in PoMTP). These conserved cysteine residues probably form disulphide bridges and are important for the general structures and functions of metalloproteases (Stocker *et al.*, 1995 and Rawlings and Barrett, 1995). The pattern of disulphide loop in zinc-binding domain is apparently conserved in astacins, reprolysins and pappalysins subfamilies of the metzincin, but not in serralysins and matrixins. Only two disulphide loops are well conserved through out the astacins members, although diverse patterns are observed in reprolysins and pappalysins. These observations suggest that the eucolysins subfamily possibly more closely relates to the astacins than to the serralysins subfamily (Joh *et al.*, 2004). Our fibrinolytic enzyme (PoFR) decreased the hematocrit percentage and prolonged TT and PT duration in thrombus induced mice (Table 5). This indicated the strong thrombolytic activity of the fibrinolytic enzyme PoFR may be due to the anticoagulant capacity or fibrin clot degradation.

```
  1 atgttgcgctccatcctgttaattgcactctcctgctttgcctacgtgctggggagctca
    M  L  R  S  I  L  L  I  A  L  S  C  F  A  Y  V  L  G  S  S
 61 cggagacccaggcaaagggtttgcgggtcgcaaccaagtcaagaaagtatccaaagggcg
    R  R  P  R  Q  R  V  C  G  S  Q  P  S  Q  E  S  I  Q  R  A
121 gaggcccattttgatggccttagcagcaacgtcagcctgaaaaaagagagttctttcacc
    E  A  H  F  D  G  L  S  S  N  V  S  L  K  K  E  S  S  F  T
181 gtcacagtgcactgccatgtcgtcagccagaattccacggtccgtggccccaacatcccc
    V  T  V  H  C  H  V  V  S  Q  N  S  T  V  R  G  P  N  I  P
241 gattcccaaattacggaccaaatccaagtcttgaatgatgcatacgcgtactcgaacttt
    D  S  Q  I  T  D  Q  I  Q  V  L  N  D  A  Y  A  Y  S  N  F
301 tcatttgagcaagcgggtaccacacgtacagtcaacgctacatggtttggcaaggccgaa
    S  F  E  Q  A  G  T  T  R  T  V  N  A  T  W  F  G  K  A  E
361 ccggagagtatataccaagatgaaatgaattcggccctgcgaaagggaaaagccgctgct
    P  E  S  I  Y  Q  D  E  M  N  S  A  L  R  K  G  K  A  A  A
421 ctaaacatttacagcgtagggtttacccctggttactactacgggcaactaggatacgca
    L  N  I  Y  S  V  G  F  T  P  G  Y  Y  Y  G  Q  L  G  Y  A
481 acgttcccatttgcacatcgctacagtccaaaggatgacggtgttgtgatccattacccc
    T  F  P  F  A  H  R  Y  S  P  K  D  D  G  V  V  I  H  Y  P
541 tcggttccgggggggcaacttggatacatacaacctcgggatgaccgcaacgcacgaagtc
    S  V  P  G  G  N  L  D  T  Y  N  L  G  M  T  A  T  H  E  V
601 ggtcactggtttggcctcaaccacaccttccaaggaggttgcgacgttcctggaccctat
    G  H  W  F  G  L  N  H  T  F  Q  G  G  C  D  V  P  G  P  Y
      Zinc-binding domain
661 gttgatgatacccccctctgactcgttcgaagggtttggatgccccgttggtcgtgacact
    V  D  D  T  P  S  D  S  F  E  G  F  G  C  P  V  G  R  D  T
721 tgcgacggcgaaggctttgaccccatccatttctacatggactacaccgatgatgagtgc
    C  D  G  E  G  F  D  P  I  H  F  Y  M  D  Y  T  D  D  E  C
                                 Met-turn
781 atgaccgagttcaccgaagggcagggtcagcgcatgagagggccctctgagtcgtacgaa
    M  T  E  F  T  E  G  Q  G  Q  R  M  R  G  P  S  E  S  Y  E
841 gcc 843
    A
```

Fig. (8): Nucleotides sequence analysis of fibrinolytic enzyme gene (PoFR-cDNA) and deduced amino acid sequence from the mycelium of P. ostreatus, the sequence extends, 843 nucleotides in length. The translation products of the fibrinolytic gene are shown below the nucleotide sequence. The putative signal peptide is shown in underlined and bold.

Table (5): In vivo assay thrombolysis of fibrinolytic enzyme purified from P. ostreatus mycelium using thrombus induced mice.

Mice	Groups								
	Healthy untreated (control)			Mice injected with thrombin			Mice injected with thrombin and treated with PoFR enzyme		
	Hematocrit (%)	TT (Sec)	PT (Sec)	Hematocrit (%)	TT (Sec)	PT (Sec)	Hematocrit (%)	TT (Sec)	PT (Sec)
M1	33.3	6.3	12.3	48.3	4.5	10.7	35.2	6.0	11.9
M2	34.5	7.2	12.5	44.5	5.0	10.5	32.0	6.5	12.0
M3	32.3	8.0	11.9	46.0	4.3	10.0	36.5	8.2	11.8
FM4	35.0	7.6	12.7	44.0	4.8	10.3	27.3	7.5	12.3
FM5	33.1	8.3	12.9	43.5	3.7	10.6	32.5	7.9	12.5

M: Male **FM:** Female

(A)

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P.osteratus(PoFR)   MLRSILLIALSCFAYVLGSSRRPRQRVCGSQPSQESIQRAEAHFDGLSSNVSLKKESSFT 60
PoMTP(AAU94648.1)   MLRSILLIALSCSAYVLGSTPRPRQRVCGSQPSQESIQRAEAHFDGLRSNVSLKKESSFT 60
                    ************ ******: ************************* ************

P.osteratus(PoFR)   VTVHCHVVSQNSTVRGPNIPDSQITDQIQVLNDAYAYSNFSFEQAGTTRTVNATWFGKAE 120
PoMTP(AAU94648.1)   VAVHWHVVSQNSTVRGGNIPDSQITDQIQVLNDAYAYSGFSFELAGTTRTVNATWFGKAE 120
                    *:** ********** ********************.**** ***************

P.osteratus(PoFR)   PESIYQDEMNSALRKGKAAALNIYSVGFTPGYYYGQLGYATFPFAHRYSPKDDGVVIHYP 180
PoMTP(AAU94648.1)   PESIYQDEMKAALRKGGAAALNIYSVGFTSGYYYGLLGYATFPFAYGYSPKDDGVVIHYA 180
                    *********::***** ************.***** *********: ************.
                                Zn-binding domain
P.osteratus(PoFR)   SVPGGNLDTYNLGMTATHEVGHWFGLNHTFQGGCDVPGPYVDDTPSDSFEGFGCPVGRDT 240
PoMTP(AAU94648.1)   SVPGGNLDTYNLGMTATHEVGHWFGLYHTFQGGCDEPGDYVDDTPSESYEAFGCPVGRDT 240
                    ************************* ******** ** *******:*:*.*********
                             Met-turn
P.osteratus(PoFR)   CDGEGFDPIHFYMDYTDDECMTEFTEGQGQRMRGPSESYEA--------- 281
PoMTP(AAU94648.1)   CDGEGFDPIHNYMDYTDDECMTEFTEGQGQRMREQSWTYRGVEARVAAPV 290
                    ********** ********************** * :*..
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(B)

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PoFR              HEVGHWFGLNHTFQ 14
AAU94648.1        HEVGHWFGLYHTFQ 14
CAB63909.1        HEVGHWFGLFHTFQ 14
                  ********** ****
```

(C)

```
PoFR              HFYMDYTDDEC 11
AAU94648.1        HNYMDYTDDEC 11
CAB63909.1        HNYMSYRQDRC 11
                  * **.* :*.*
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Fig. (9): (A) Multiple sequences alignment the deduced amino acid sequence of fibrinolytic gene (PoFR) with accession number (BAI87841.1) isolated from mycelium of P. ostreatus with that published in GenBank with accession number AAU94648.1 isolated from fruit bodies of P. ostreatus. The Zn-binding domain and Met-turn sequence were underlined. Four Cys residues involved in the formation of two disulfide bridges were shaded. The difference of amino acids between our gene and PoTMP were bold. (*) means identity, (:) conserved amino acids, (.) semi-conserved amino acids and (--) no identity. (B and C) Alignment of consensus sequence and Met-turn between PoFR, PoMTP and (CAB63909.1) from Metarhizium anisopliae var. anisopliae. Alignment was performed using CLUSTALW 2.0 software (http://www.ebi.ac.uk/Tools/ clustalw2/index.html).

Kim and Lee (2006) reported that the *in vivo* assay of *Umbilicaria esculenta* ethanolic extract revealed that it could be a potent source of antithrombotic agent which might due to antiplatelet activity. They also reported that mice tail bleeding time was significantly prolonged by addition of the extract. It also alters the coagulation parameters such as activated partial thromboplastin time (APTT), prothrombin time (PT) and thrombin time (TT) in rat platelets. In this concern, Peng *et al*. (2005) carried out *in vivo* assay on nattokinase (NK) from *B. natto* and found that the enzyme not only directly cleaves cross-linked fibrin but also activates the production of t-PA resulting in transformation of inactive plasminogen into active plasmin. Sumi *et al*.

(1990) showed that the blood clots in the dogs that received nattokinase NK capsules completely dissolved within 5 h of treatment and normal blood circulation was restarted. Fujita *et al.* (1995) found that rats treated with NK recovered 62% of the arterial blood flow whereas those treated with plasmin regained just 15.8%.

REFERENCES

Ahn, M. Y., Hahn B. S., Ryu K. S., Kim J. W., Kim I. and Kim Y. S. (2003). Purification and characterization of a serine protease with fibrinolytic activity from the dung beetles, *Catharsius molossus*. Thrombosis Research, vol. 112, pp. 339-347, 2003.

Ang, H. Y., Choi S. S., Chi W. J., Kim J. H., Kang D. K., Chun J., Kang S. S. and S. K. Hong (2005). Identification of the sprU gene encoding an additional sprT homologous trypsin-type protease in *streptomyces griseus*. Journal of Microbiology and Biotechnology, vol. 15, pp. 1125-1129.

Bradford M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding", Analytical Biochemistry, vol. 72, pp. 248-254.

Choi H.S. and Shin H. H. (1998). Purification and partial characterization of a fibrinolytic protease in *Pleurotus ostreatus*. Mycologia, vol. 90, pp. 674-679, 1998.

Choi N. S., Yoo K. H., Hahm J. H., Yoon K. S., Chang K. T., Hyun B. H., Maeng P. J. and Kim S. H. (2005). Purification and characterization of a new peptidase, bacillopeptidase DJ-2, having fibrinolytic activity: Produced by *Bacillus* sp. DJ-2 from Doen-Jang. Journal of Microbiology and Biotechnology, vol. 15, pp. 72-79.

Collens D. and Lijnen H. R. (1991). Basic and clinical aspects of fibrinolysis and thrombosis. Blood, vol. 78, pp. 3114-3124.

Datta G., Dong A., Witt J. and Tu A. T. (1995). Biochemical characterization of basilase, a fibrinolytic enzyme from *Crotalus basiliscus*", Archives of Biochemistry and Biophysics, vol. 317, no. 2, pp. 365-373.

De-Simone S. G., Correa-Netto C., Antunes O. A. C., De-Alencastro R. B. and Silva F. P. (2005). Biochemical and molecular modeling analysis of the ability of two paminobenzamidine - based sorbents to selectively purify serine proteases (fibrinogenases) from snake venoms", Journal Chromatography B Analyte Technology Biomed. Life Science, vol. 822, pp. 1-9.

Fujita M., Hong K., Ito Y., Fujii R., Kariya K. and Nishimuro S. (1995). Thrombolytic effect of nattokinase on a chemically induced thrombosis model in rat", Biological and Pharmaceutical Bulletin, vol. 18, no. 10, pp. 1387-1391.

Harlan J. M. and Harker L. A. (1981). Haemostasis, thrombosis and thromboembolic disorder", Medical Clinical of North America, vol. 65, pp. 855-857, 1981.

Healy V., O'Connell J., McCarthy T. V. and Doonan S. (1999). The lysine-specific proteinase from *Armillaria mellea* is a member of a novel class of metalloendopeptidases located in Basidiomycetes. Biochemical and Biophysical Research Communications, vol. 262, pp. 60-63.

Huang Y., Peng K., Su J., Huang Y., Xu Y. and Wang S. (2007). Different effects of homocysteine and oxidized low density lipoprotein on methylation status in the promoter region of the estrogen receptor α

gene. Acta Biochimica et Biophysica Sinica, vol. 39, pp. 19-26.

Jia Y. H., Jin Y., Lu Q. M. and D. S. Li. (2003). A novel serine protease with kinin-releasing and fibrinogenolytic activity from *Trimeresurus jerdonii* venom. Acta Biochim. Biophys. Sin. Vol. 35, pp. 689-694.

Joh H., Kim B. G., Kong W. S., Yoo Y. B., Kim N. K., Park H. R., Cho B. G. and Lee C. S. (2004). Cloning and developmental expression of a family metalloprotease cDNA from oyster mushroom *Pleurotus ostreatus*. FEMS Microbiology Letters, vol. 239, pp. 57-62.

Jiang W. and Bond J. S. (1992). Families of metalloendopeptidases and their relationships. FEBS Letters, vol. 312, pp. 110-114.

Kim M. S. and Lee K. (2006). Antithrombotic activity of methanolic extract of *Umbilicaria esculenta*. Journal of Ethnopharmacology, vol. 105, pp. 342-345.

Kim S., Kim J. E., Choi B. S., Park S. E., Saptoka K., Kim S., Lee H. H., Kim C. S., Park Y., Kim M. K., Kim Y. S. and Kim S. J. (2008). Purification and characterization of fibrinolytic metalloprotease from *Perenniporia fraxinea* mycelia. Mycological Research, vol. 112, pp. 990-998.

Kim J. H. and Kim Y.S. (1999). A fibrinolytic metalloprotease from the fruiting bodies of an edible mushroom, *Armillariella mellea*. Bioscience Biotechnology, and Biochemistry, vol. 63, pp. 2130-2136.

Kim J. S., Kumar S., Park S. E., Choi B. S., Kim S., Nguyen T. H., Kim C. S., Choi H. S., Kim M. K., Chun H. S., Park Y. and Kim S. J. (2006). A fibrinolytic enzyme from the medicinal mushroom *Cordyceps militaris*. Journal of Microbiology, vol. 44, pp. 622-631.

Laemmli U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, vol. 227, pp. 680-685.

Lee S. Y., Kim J. S., Kim J. E., Sapkota K., Shen M. H., Kim S., Chun H. S., Yoo J. C., Choi H. S., Kim M. K. and Kim S. J. (2005). Purification and characterization of fibrinolytic enzyme from cultured mycelia of *Armillaria mellea*. Protein Expression and Purification, vol. 43, no. 1, pp. 10-17.

Liu G., Xing J. M., Chang T. S., Ma Z. Y. and Liu H. Z. (2005). Optimization of nutritional conditions for nattokinase production by *Bacillus natto* NLSSE using statistical experimental methods. Process Biochemistry, vol. 40, pp. 2757-2762.

Mihara H., Sumi H., Yoneta T., Mizumoto H., Ikeda R. and Seiki M. (1991). A novel fibrinolytic enzyme extracted from the earthworm, *Lumbricus rubellus*. Japanese Journal of Physiologyl, vol. 41, pp. 461-472.

Nonaka T., Naoshi D., Yohichi H., and Koji T. (1997). Amino acid sequences of metalloendopeptidases specific for acyl-lysine bonds from *Grifola frondosa* and *Pleurotus ostreatus* fruiting bodies. Journal of Biological Chemistry, vol. 272, pp. 30032-30039.

Peng Y., Yang X. and Zhang Y., (2005). Microbial fibrinolytic enzymes: an overview of *source*, production, properties, and thrombolytic activity *in vivo*. Applied and Environmental Microbiology, vol. 69, pp. 126-132.

Poindexter K., Nelson N., DuBose R. F., Black R. A. and Cerretti D. P. (1999). The identification of seven metalloproteinase-disintegrin (ADAM) genes from genomic libraries. Gene, vol. 237, pp. 61-70.

Prasad J. K., Mohan S. V., Rao R. S., Pati B. R. and Sarma P. N. (2005). Laccase production by *Pleurotus ostreatus* 1804: optimization of submerged culture conditions by Taguchi DOE methodology. Biochemical Engineering Journal, vol. 24, pp. 17-26.

Rawlings N. D. and Barrett A. J. (1995). Evolutionary families of metalloporteases. Methods in Enzymology, vol. 248, pp. 183-228.

Shen H. M., Kim J. S., Sapkota K., Park S. E., Choi B. S., Kim S., Lee H. H., Kim C. S., Chun H. S., Ryoo C. I. and Kim S. J. (2007). Purification, characterization and cloning of fibrinolytic metalloprotease from *Pleurotus ostreatus* mycelia. Journal of Microbiology and Biotechnology, vol. 17, no. 8, pp. 1271-1283.

Shin H. H. and Choi H. S. (1999). Purification and characterization of metalloproteases from *Pleurotus sajorcaju.* Journal of Microbiology and Biotechnology, vol. 9, pp. 675-678.

Stocker W., Grams F., Baumann U., Reinemer P., Gomis-Ruth F. X., McKay D. B. and Bode W. (1995). The metzincins-topological and sequential relations between the astacins, adamalysins, serralysins, and matrixins (collagenases) define a super family of zinc-peptidases. Protein Science, vol. 4, pp. 823-840.

Stricklin G. P and Hibbs M. S. (1988). Biochemistry and physiology of mammalian collagenases. pp. 187. In M. E. Nimni (ed.), Collagen: Volume I, Biochemistry, CRC Press, Boca Raton, FL.

Sumi H., Hamada H., Nakanishi K. and Hiratani H. (1990). Enhancement of the fibrinolytic activity in plasma by oral administration of nattokinase. Acta Haematologica, vol. 84, no 3, pp. 139-143.

Sumi H., Nakajima N. and Mihara H. (1992). Fibrinolysis relating substances in marine creatures. Comparative Biochemistry and Physiology, vol. 102, pp. 163-167.

Vallee B. L. and Auld D. S. (1990). Active-site zinc ligands and activated H_2O of zinc enzymes. Proceedings of the National Academy of Sciences, vol. 87, pp. 220-224.

Voet D. and Voet J. G. (1990). Biochemistry. pp. 1087-1095, 2nd Ed. John Willey and Sons, New York, U.S.A.

Wang C., Ji B. P., Li B., Nout R., Li P. L., Ji H. and Chen L. F. (2006). Purification and characterization of a fibrinolytic enzyme of *Bacillus subtilis* DC33, isolated from Chinese traditional Douchi. Journal of Industrial Microbiology and Biotechnology, vol. 33, pp. 750-758.

Wang F., Wang C. and Li M. (2005). Crystal structure of earthworm fibrinolytic enzyme component B: A novel, glycosylated two chained trypsin. Journal of Molecular Biology, vol. 348, pp. 671-685.

Woessner J. F. (1991). Matrix metalloproteinases and their inhibitors in connective tissue remodeling. FASEB Journal, vol. 5, pp. 2145-2154.

Yuk D. Y., Ryu C. K., Hong J. T., Chung K. H., Kang W. S., Kim Y., Yoo H. S., Lee M. K., Lee C. K. and Yung Y. P. (2000). Antithrombotic and antiplatelet activities of 2-chloro-3-[4-(ethylcarboxy)-phenyl]-amino-1,4-naphthoquinon (NQ12), a newly synthesized 1,4-naphthoquinon derivative. Biochemical Pharmacology, vol. 60, no. 7, pp. 1001-1008.

الملخص العربي

التوصيف الجزيئى والبيوكيميائى للانزيم المحلل للفيبرين المُنقى من بلوروتس أوستراتس

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تم تصميم نظام يعتمد على العوامل المختلفة للبيئات لعمل ١٨ وسط غذائى يحتوى على مكونات مختلفه و ظروف مناسبه لانتاج الأنزيم المحلل للفيبرين بكميات كبيره من فطرة عيش الغراب الغذائيه بلوروتس اوستراتس. تم تقدير نشاط الانزيم المحلل للفيبرين و البروتين و النشاط البروتينى و نسبة F/P و الانشطه المتخصصه فى جميع الاوساط الغذائيه المستخدمه. تم تقدير أعلى نسبه لنشاط الانزيم المحلل للفيبرين الى النشاط البروتينى F/P فى الوسط الغذائى رقم ٢ و التى تتكون من (جرام/١٠٠ملى) فيبرين ٠.٢ و مستخلص الخميرة ١.٥ و الجلوكوز ١.٠ و كبريتات الماغنيسيوم ٠.١ و ثنائى البوتاسيوم هيدروجين فوسفات ٠.٢ و كان الاس الهيدروجينى ٦ و درجة الحرارة ٣٥ سليزيوس وتم التحضين لمدة ٧ أيام. تم تقدير أعلى نسبه لنشاط البروتينى ويحتوى على أقل F/P فى الوسط الغذائى رقم ٦ و الذى يتكون من (جرام/١٠٠ملى) فيبرين ٠.٣ و مستخلص الخميرة ١.٠ و الجلوكوز ١.٠ و كبريتات الماغنيسيوم ٠.١ و ثنائى البوتاسيوم هيدروجين فوسفات ٠.٢ و كان الاس الهيدروجينى ٧ و درجة الحرارة ٤٥ سليزيوس وتم التحضين لمدة ٧ أيام. تمت التنقيه الكامله للانزيم المحلل للفيبرين من الغزل الفطرى لفطرة البلوروتس اوستراتس عن طريق الفصل الكروماتوجرافى باستخدام DEAE-cellulose و Sephadex G-100. تم تمرير الانزيم المحلل للفيبرين النقى على جهاز الفصل الكهربى و تم تحديد الوزن الجزيئى للانزيم النقى و هو ٤٤ كيلو دالتون. تم توصيف حركية الانزيم المحلل للفيبرين النقى مثل تأثير الاس الهيدروجينى، درجة الحرارة، مثبطات البروتين و أيونات المعادن. وجد ان الاس الهيدروجينى الأمثل لنشاط الانزيم المحلل للفيبرين هو ٦ و جد ان درجة الحرارة المثلى للانزيم المحلل للفيبرين هى ٣٥ درجة سيلزيوس حيث ان نشاط الانزيم ثابت عند درجة الحرارة تتراوح من ٣٠-٤٠ درجه. وجد ان مادتى EGTA و EDTA يعتبروا مثبطات قويه لنشاط الانزيم المحلل للفيبرين ووجد ايضا ان نشاط الانزيم يثبط كليا بواسطة مثبطات البروتيز مثل TLCK بينما مثبطات الاسبرتك مثل pepstatin A لها تأثير ضعيف على نشاط الانزيم. الانزيم المحلل للفيبرين النقى من بلوروتس اوستراتس تم توصيفه على انه serine metalloprotease. تم عزل الحمض النووى الريبوزى الكلى من الغزل الفطرى لفطره عيش الغراب ووجد ان cDNA الناتج حجمه حوالى ٨٤٥ قاعدة مزدوجة تم مقارنة التتابع الجينى للجين المعزول من الغزل الفطرى للبلوروتس اوستراتس بالجين المعزول من الجسم الثمرى للبلوروتس اوستراتس ووجد ان نسبة التشابه هى ٩٥%. تم مقارنة تتابع الأحماض الأمينيه للجين المعزول من الغزل الفطرى للبلوروتس اوستراتس الأحماض الأمينيه للجين المعزول من الجسم الثمرى للبلوروتس اوستراتس ووجد ان نسبة التشابه هى ٩٠%. كماأوضحت الأختبارات ان الانزيم المحلل للفيبربن من البلوروتس اوستراتس له قدرة عاليه على تحليل الجلطات (الفيبرين) فى ذكور و اناث الفئران و قد تم اثبات ذلك عن طريق القيم التى توصلنا اليها للعوامل المضادة للتجلط مثل TT و PT و كذلك لعوامل التجلط مثل نسبة الهيماتوكريت. و بذلك تم اثبات أن PoFR له قدرة عاليه كمضاد للتجلط وكذلك كمذيب للجلطات.