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Herinase: A Novel Bi-functional Fibrinolytic Protease from the Monkey Head Mushroom, *Hericium erinaceum*

Bong-Suk Choi · Kumar Sapkota · Jun-Hui Choi · Chang-ho Shin · Seung Kim · Sung-Jun Kim

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Abstract Herinase, a new bi-functional fibrinolytic metalloprotease, was purified from a medicinal and edible mushroom *Hericium erinaceum*. The enzyme was monomeric with a molecular mass of 51 kDa. Analysis of fibrin zymography showed an active band with a similar molecular mass. The N-terminal sequence of herinase VPSSFRTTITDAQLRG was highly distinguished from known fibrinolytic enzymes. Moreover, the enzyme activity was strongly inhibited by EDTA and EGTA, indicating that herinase is a metalloprotease. Herinase exhibited high specificity for the substrate t-PA followed by plasmin. The K_m and V_{max} values for H-D-Ile-Pro-Arg-PNA were found to be 4.7 mg and 26.7 U/ml respectively. Similarly, fibrin plate assays revealed that it was able to degrade fibrin clot directly and also able to activate plasminogen. Herinase provoked a rapid degradation of fibrin and fibrinogen α chains and slower degradation of γ chains. It had no activity on the β chains of fibrin and fibrinogen. This result suggests that herinase could possibly contain higher amount of α-fibrinogenase. The activity of herinase was stimulated by metal ions such as Ca^{2+}, Mg^{2+}, and Mn^{2+}, but inhibited by Cu^{2+}, Fe^{2+}, and Zn^{2+}. Herinase exhibited maximum activity at 30 °C and pH 7.0. These results demonstrate that herinase could be a novel fibrinolytic enzyme.

Keywords Bi-functional enzyme · Fibrinolysis · *Hericium erinaceum* · Metalloprotease · Thrombosis

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Introduction

Cardiovascular disease is the leading cause of death throughout the world and is viewed as a global epidemic. According to the World Health Organization, there will be about 20 million deaths from this devastating disease in 2015. It is a substantial and rapidly growing problem around the world, affecting people from all socioeconomic backgrounds. Thrombosis—the abnormal localized blood clot inside a blood vessel—is one of the main causes of cardiovascular diseases. Fibrin is the main structural component of the thrombus, and its improper deposition in the blood vessels usually induces thrombosis. Fibrin is formed from fibrinogen, a glycoprotein that consists of pairs of three different polypeptide Aα, Bβ, and γ chains via proteolysis by the enzyme thrombin [1, 2]. Fibrin clot lysis is a crucial mechanism mediated by plasmin, a serine protease that hydrolyzes the insoluble fibrin fiber at specific sites. Plasmin is derived from plasminogen by plasminogen activators. Fibrinolytic agents, also referred to as plasminogen activators, are most powerful weapons in treating thrombosis. They have the unique ability to dissolve the catastrophic blood clots through the activation of intrinsic components of the fibrinolytic system. Currently, several fibrinolytic agents, including anticoagulants, antiplatelet, and direct thrombolytics, are available for the treatment of thrombosis, but all these agents have certain limitations [3]. Moreover, despite many years of research, an ideal thrombolytic agent has yet to be developed [4]. Therefore, there is a need to search for effective and safe fibrinolytic agents with novel mechanisms of action that can dissolve a thrombus reliably. In recent years, fibrinolytic proteases found in food sources have received significant attention due to their efficiency and safety. Since ancient times, mushrooms have been used not only as a source of food but also for medicinal purposes. They are a rich source of variety of bioactive compounds including fibrinolytic proteases with a remarkable effect on prevention of cardiovascular diseases [5, 6]. Recently, we and others have attempted to identify and characterize the fibrinolytic proteases from several edible or medicinal mushrooms including *Perenniporia fraxinea*, *Grifola frondosa*, *Flammulina velutipes*, *Fomitella fraxinea*, *Pleurotus ostreatus*, *Armillaria mellea*, *Cordyceps militaris*, and *Paecilomyces tenuipes* [5, 7].

Hericium erinaceum (also called lion's mane or monkey head mushroom) is a unique and beautiful white fungus, which has long been used as a traditional medicine and healthy food in East Asian countries [8, 9]. *H. erinaceum* contains various bioactive substances such as polysaccharides, proteins, lectins, lipids, hericenone, erinacol, erinacine, and terpenoids, and hence was reported to exert antitumor [8], anti-inflammatory [10, 11], anti-skin aging [12], immunomodulatory [13–15], and neuroprotective [9, 16–18] effects. Nonetheless, the fibrinolytic protease from *H. erinaceum* has not yet been reported. In the present study, we attempted to purify fibrinolytic protease from this mushroom and analyze its biochemical properties. We demonstrate the remarkable fibrinolytic ability of this novel bifunctional protease from *H. erinaceum*.

Materials and Methods

Materials

The fruiting bodies of *H. erinaceum* were obtained from Korean Mushroom Company (Suwon, Republic of Korea). Human fibrinogen, thrombin, plasmin, acrylamide, citrate monohydrate, sodium chloride, trizma base, trizma HCl, phenylmethylsulfonyl fluoride (PMSF), tosyllysine chloromethyl ketone (TLCK), aprotinin, tosylphenylalanine chloromethyl keptone (TPCK), ethyleneglycolbis-(2-aminoethyl)-*N*,*N*,*N*′,*N*′ tetraacetic acid

(EGTA), ethylenediaminetetraacetic acid (EDTA), carboxymethyl (CM)-cellulose, and diethylaminoethyl (DEAE)-cellulose were purchased from Sigma-Aldrich (St. Louis, MO, USA). Paranitroaniline (pNA) chromogenic substrates were obtained from Chromogenix (Milan, Italy). Agarose was obtained from Invitrogen (Carlsbad, CA, USA). PageRuler^{TM} color protein marker was purchased from Fermentas (Hanover, MD, USA). Sephadex G-75 and HiLoad 16/60 Superdex 75 were obtained from Pharmacia (Uppsala, Sweden). Other reagents were special grade as commercially available.

Enzyme Purification

All procedures were carried out at 4 °C unless otherwise stated. Protein concentration was measured by using the BCA protein assay kit (Pierce, Rockford, IL, USA) with bovine serum albumin as a standard. The crude enzyme from the fruiting bodies of *H. erinaceum* was prepared as described previously [7]. The crude enzyme was first applied to a CM-cellulose column (3×10 cm) equilibrated with 10 mM citrate–NaOH (pH 6.8) buffer. The column was eluted at a flow rate of 0.1 ml/min. Fractions exhibiting fibrinolytic activity were pooled and loaded onto a DEAE-cellulose column (3×10 cm) pre-equilibrated with 10 mM Tris–HCl (pH 7.4) buffer then eluted with linear gradient of 0–1.0 M NaCl at a flow rate of 0.1 ml/min. The active fractions were collected and concentrated using a Vivaspin 20 (Sartorius Stedim Biotech, Gottingen, Germany). The concentrated sample was applied to a Sephadex G-75 column (1.0 cm×63 cm) equilibrated with 10 mM Tris–HCl buffer (pH 7.4) containing 0.15 M NaCl and then eluted at a flow rate of 1.0 ml/min. The active fractions were pooled and concentrated. The fractions were further purified by fast protein liquid chromatography (FPLC) using a HiLoad 16/60 Superdex 75 column with a flow rate of 0.5 ml/min. Fractions with fibrinolytic activities were pooled and concentrated and used as the purified enzyme (named as herinase). The purity of the enzyme was confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and the activity of the enzyme was assessed by the fibrinolytic assays as described below.

Enzyme Activity Assay

Enzyme activity was measured at room temperature in 96-well plates (SPL Life Sciences) using a microplate reader (Molecular Devices, Sunnyvale, CA, USA) according to the previously described methods [7, 19]. The reaction mixture composed of 40 μl of 1 % human fibrinogen, 10 μl of 5 U/ml thrombin, and enzyme sample/column fraction (10 μl) [all dissolved in Tris buffer (50 mM Tris–HCl, pH 7.4) containing 0.1 M NaCl] was incubated at 37 °C for 10 min. Plasmin was used as a control. Readings were taken at wavelength of 350 nm. One unit of enzyme activity was defined as the amount of enzyme causing conversion of 1 μM of substrate per minute per milligram of protein at 37 °C.

Enzyme Analysis

To determine the molecular mass and homogeneity of the purified enzyme, SDS–PAGE was performed according to the method of Laemmli [20]. The protein samples were mixed with 5× SDS–PAGE sample buffer [60 mM Tris–HCl (pH 6.8), 25 % glycerol, 2 % SDS, 14.4 mM β-mercaptoethanol, 0.1 % bromophenol blue]. The mixture was heated at 100 °C for 5 min and electrophoresed in 12 % polyacrylamide gel. The protein bands were visualized by staining the gel with Coomassie Brilliant Blue R-250. Molecular weight was measured with standard marker (Fermentas Life Sciences).

The protease was also analyzed by fibrin zymography according to the method of Kim et al. [21]. Resolving gel solution (12 %) contained 0.12 % (w/v) fibrinogen prepared in a total volume of 10 ml and centrifuged to remove insoluble impurities which were induced when SDS stock solution was mixed. Thrombin solution (1 U/ml) and TEMED (N,N,N^0,N^0-tetramethylethylenediamine) were added to the gel solution in final concentrations of 0.1 U/ml and 0.028 % (v/v), respectively. The purified enzyme was electrophoresed on a fibrin gel. The gel was then washed in 2.5 % Triton X-100 solution, incubated in zymogram reaction buffer [30 mM Tris (pH 7.4) containing 200 mM NaCl, 10 mM $CaCl_2$, and 0.02 % NaN_3] at 37 °C overnight, stained with Coomassie Brilliant Blue R-250, and then destained.

Determination and Analysis of N-terminal Amino Acid Sequence of the Enzyme

To determine the N-terminal amino acid sequence, the purified enzyme was subjected to SDS–PAGE and electroblotted onto a polyvinylidene difluoride membrane (PVDF) (Millipore, Watford, UK) using a Minitransblot electroblotting system (Bio-Rad, Hercules, CA, USA). The membrane was stained with Coomassie Brilliant Blue R-250, followed by destaining. The desired protein band was excised from the membrane, and the amino acids of the N-terminal sequence were determined using an automatic DNA sequencer (ABI PRISM 377, Perkin Elmer, USA) at the Korea Basic Science institute, Daejeon, Republic of Korea. The homology between the purified enzyme and other proteinases was performed using the NCBI "BLAST" search program.

Activity of the Enzyme Towards Chromogenic Substrates

The hydrolytic activity of the purified enzyme was analyzed using different synthetic chromogenic substrates in a 96-well plate reader (Molecular Devices). The synthetic chromogenic substrates used were S-2765, S-2238, S-2251, S-2288, and S-2444. The reaction was initiated by adding the purified enzyme (1 μg/175 μl of 50 mM Tris–HCl, pH 7.4) containing 0.1 M NaCl with 25 μl of 4 mM chromogenic substrates. After incubation for 5 min at 37 °C, the amount of released *p*-nitroaniline was determined by measuring the change in absorbance at 405 nm. The K_m and V_{max} of the purified enzyme were determined with different concentrations of H-D-Ile-Pro-Arg-pNA as a substrate.

Fibrinolytic Assays

Fibrinolytic activity was determined by slightly modified fibrin plate method [22] using both plasminogen-free and plasminogen-rich plates. Plasminogen-free fibrin plate was prepared by mixing agarose (1.0 %, w/v) in Tris buffer (50 mM Tris–HCl, pH 7.4) containing 0.1 M NaCl (55 °C) with fibrinogen (1 %) (1.0 mg/ml), and human thrombin (0.5 U/ml) dissolved in same buffer. Plasminogen-rich plate was supplemented with 5 U of plasminogen. The plates were allowed to set at room temperature for 1 h. Then, 10 μl of sample solution (1.0 μg) was carefully applied to the wells made in the plate. The plates were incubated for 12 h at 37 °C.

The fibrinolytic activity was also analyzed as described by Datta et al. [23], with slight modification. Seventy-five microliters of 1 % human fibrinogen in Tris buffer [50 mM Tris–HCl (pH 7.4) containing 0.1 M NaCl] was mixed to 5 μl of human thrombin (0.25 U). The fibrin clot was allowed to form at room temperature for 1 h. Then, 20 μl (1 μg) of purified enzyme or plasmin was added to the fibrin clot and incubated at 37 °C for indicated time periods. The

reaction was terminated by the addition of 10 μl of denaturing buffer (1 M urea, 4 % SDS, and 4 % β-mercaptoethanol) and analyzed by 12 % SDS–PAGE.

Fibrinogenolytic Assay

Fibrinogenolysis was determined according to the method of Matsubara et al. [24]. The reaction mixture containing 80 μl of 1 % human fibrinogen in 50 mM Tris–HCl (pH 7.4) containing 0.1 M NaCl was incubated with 10 μl (1 μg) of a purified enzyme or plasmin at 37 °C for indicated time periods. The reaction was stopped by adding 10 μl of denaturing buffer. A portion of the reaction mixture was withdrawn and analyzed by 12 % SDS–PAGE.

Determination of Optimum pH and Temperature on Fibrinolytic Activity

The optimal pH for the fibrinolytic activity of the enzyme was determined within a pH range of 2.0–11.0. One microgram of the enzyme solution was added to 90 μl of 50 mM glycine–HCl, citric–NaOH, Tris–HCl, and glycine–NaOH buffer system. The reaction mixtures were incubated for 1 h and the enzyme activities were measured by the enzyme activity assay as described above.

The optimal temperature for fibrinolytic activity was determined by measuring residual activity after the incubation of 1.0 μg of herinase in 90 μl of 50 mM sodium phosphate buffer at different temperatures (10–70 °C) for 1 h.

Enzyme Inhibition Assay

To determine the nature of the enzyme, the effects of protease inhibitors were assessed using PMSF, TLCK, TPCK, EDTA, EGTA, and aprotinin. The purified enzyme (1.0 μg) was pre-incubated in a final concentration of 1.0 mM PMSF and aprotinin, 2 mM TPCK, TLCK, EDTA, and EGTA in 50 mM Tris–HCl for 1 h at 37 °C. After incubation of each reaction solution (10 μl) with fibrin clot prepared from human fibrinogen and thrombin as described above in enzyme activity assay, the enzyme activity was measured by a microplate reader (Molecular Devices). The level of inhibition was expressed as a percentage of the remaining activity (with either metal ion or inhibitor) as compared to the control activity (without metal ion or inhibitor).

Effect of Metal Ions on Fibrinolytic Activity

The effects of metal ions on enzyme activity were investigated using $CaCl_2$, $CoCl_2$, $CuCl_2$, $MgCl_2$, $MnCl_2$, $FeCl_2$, and $ZnCl_2$. The purified enzyme (1.0 μg) was incubated in the absence and the presence of cations such as Ca^{2+}, Co^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+}, Fe^{2+}, and Zn^{2+} with a final concentration of 1 mM in 50 mM Tris–HCl for 1 h at 37 °C. Each enzyme solution (10 μl) was then incubated with fibrin clot prepared from human fibrinogen and thrombin as described above in enzyme activity assay at 37 °C for 10 min, and the enzyme activity was measured by a microplate reader (Molecular Devices).

Statistical Analysis

The data were expressed as the means±SD. Statistical significance was assessed with one-way analysis of variance followed by the Turkey method. Differences with p value less than 0.05 were considered statistically significant.

Results

Enzyme Purification and Analysis by SDS–PAGE and Fibrin Zymography

Herinase, a new fibrinolytic enzyme, was purified from fruiting bodies of *H. erinaceum* by combination of ion exchange chromatography with CM-cellulose and DEAE-cellulose columns (Fig. 1a, b) and gel filtration with Sephadex G-75 (Fig. 1c) and Hiload 16/60 Superdex 75 columns (Fig. 1d). As summarized in Table 1, the crude extract containing 2,692.29 mg of protein showed a specific activity of 0.41 U/mg. After final purification, 0.69 mg of enzyme was obtained from 200 g of *H. erinaceum* with a recovery of 13.7 %. The specific activity of the purified enzyme was calculated to be 220.65 U/mg of protein, which represents approximately 538.2-fold increase over the crude extract. The proteins from each purification steps were subjected to SDS–PAGE (Fig. 1e). The purified enzyme exhibited a single band with an apparent molecular mass of 51 kDa by SDS–PAGE (Fig. 2a). A strong fibrinolytic activity of the purified enzyme with similar molecular weight as estimated by SDS–PAGE was also observed in fibrin zymography (Fig. 2b).

N-Terminal Amino Acid Sequencing

The N-terminal amino acid sequence of the purified fibrinolytic enzyme was determined by automatic Edman degradation. The first 16 amino acid residues of the herinase N-terminal sequence were VPSSFRTTITDAQLRG (Table 2). In the NCBI database, no putative conserved domains were identified. However, this N-terminal sequence exhibits 62.5 % identity with the metallopeptidase from *Leishmania infantum* JPCM5 (NCBI GenBank

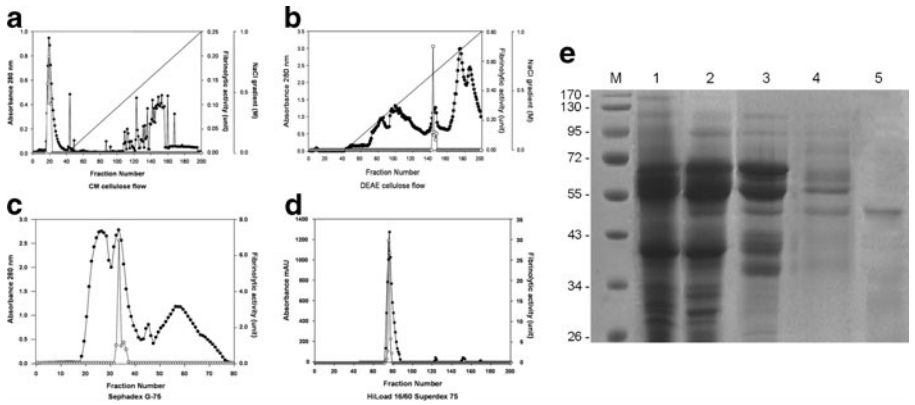

Fig. 1 Purification of herinase from *Hericium erinaceum* by column chromatography. **a** Cation-exchange chromatography of *H. erinaceum* crude proteins on CM cellulose column. The column was initially eluted with 10 mM citrate–NaOH (pH 6.8) buffer and subsequently with a linear gradient of 0–1.0 M NaCl. The flow rate was 0.1 ml/min. **b** Anion-exchange chromatogram of the collected fractions with fibrinolytic activity from (**a**) on a DEAE-cellulose column equilibrated with 10 mM Tris–HCl (pH 7.4) buffer with linear gradient of 0–1.0 M NaCl. **c** The collected fractions with fibrinolytic activity were applied to gel filtration chromatography with a Sephadex G-75 column equilibrated with the same buffer at a flow rate of 1.0 ml/min. **d** The active fractions containing fibrinolytic activity from (**c**) were subjected to FPLC HiLoad 16/60 Superdex 75 column. Fibrinolytic activity was measured by enzyme activity assay as described in "Materials and Methods". *Filled circles*—protease activity, *open circles*—fibrinolytic activity. **e** SDS–PAGE of each purification steps. *Lane M* protein marker, *1* crude sample, *2* fraction from CM cellulose column, *3* fraction from DEAE cellulose column, *4* fraction from Sephadex G-75 column, *5* fraction from HiLoad 16/60 Superdex 75 column

Table 1 Purification summary of herinase from *H. erinaceum*

Purification step	Volume (ml)	Total protein (mg)	Total protease activity (U)	Specific activity (U/mg)	Yield (%)	Purification fold
Homogenate	500	ND	ND	–	–	–
Crude extract	496	2,692.29	1,110.71	0.41	100	1
CM cellulose	100	220.53	984.24	4.46	88.6	10.9
DEAE cellulose	12.92	11.44	643.10	56.22	57.9	137.1
Sephadex G-75	7.12	1.71	183.32	107.20	16.5	261.5
HiLoad 16/60 Superdex 75	4.50	0.69	152.25	220.65	13.7	538.2

The units of activity are calculated on the basis of the plasmin standard

accession number XP-001467528). The literature survey revealed that the N-terminal amino acid sequence of the herinase is different from reported fibrinolytic enzymes isolated from other fungal strains (Table 2). These results suggest that herinase could be a distinct protease.

Hydrolytic Activity Toward Synthetic Substrates

We first analyzed the cleavage specificity of the herinase towards the synthetic chromogenic substrate for thrombin (H-D-Phe-Pip-Arg-pNA), plasmin (H-D-Val-Leu-Lys-pNA), urokinase (pyroGlu-Gly-Arg-pNA), tPA (H-D-Ile-Pro-Arg-PNA), and for factor Xa (Z-D-Arg-Gly-Arg-pNA·2HCl). The enzyme showed significantly higher hydrolytic activity toward the substrate H-D-Ile-Pro-Arg-PNA for tPA (16.7±0.5 μmol/min/mg) followed by H-D-Val-Leu-Lys-pNA for plasmin (11.2±0.4 μmol/min/mg) (Table 3), suggesting that it possesses both plasmin- and tPA-like properties. The apparent kinetic parameters (K_m and V_{max}) of herinase for H-D-Ile-Pro-Arg-PNA were found to be 4.7 mg and 26.7 U/ml, respectively.

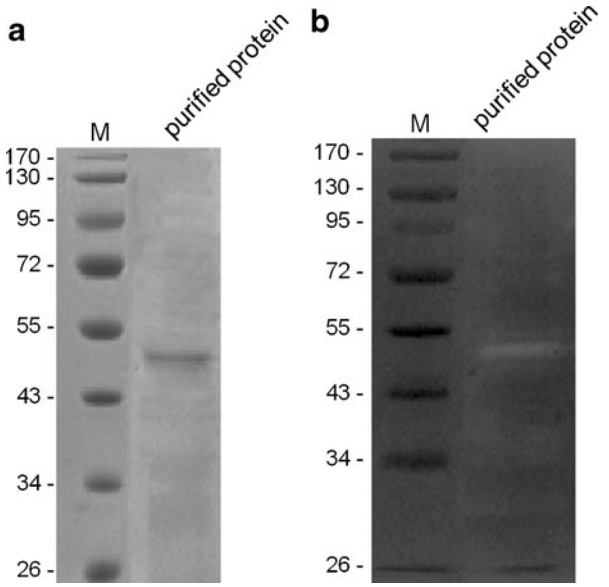

Fig. 2 Determination of purity, molecular mass, and fibrinolytic activity of herinase. **a** SDS–PAGE analysis of purified herinase. Herinase resolved from FPLC HiLoad 16/60 Superdex 75 column was electrophoresed on 12 % polyacrylamide gel under reducing conditions. **b** Fibrin zymography analysis of purified herinase. Fibrin zymography was performed on 12 % acrylamide gel containing fibrinogen and thrombin. The gels were stained with Coomassie Brilliant Blue R-250. *Lane M* protein size marker

Table 2 Comparison of N-terminal amino acid sequence of herinase

Enzyme	N-terminal amino acid sequence	Identity (%)	Molecular weight (kDa)	Reference
Hericium erinaceum (Herinase)	VPSSFRTTITDAQLRG	–	51	This work
Leishmania infantum JPCM5 (metallopeptidase)	VPSSFRTILTNGEERG	62.5	–	XP-001467528 (NCBI accession number)
Mycobacterium abscessus M94 (metallopeptidase)	AGSSFRTTVTYTFNEQ	43.8	–	EIC65812.1 (NCBI accession number)
Pleurotus ostreatus (PoFE)	ALRKGGAAALNIYSVG	6.3	32.0	[25]
Armillariella mellea (AMMP)	SLSSRFFLYTLCLSAV	18.8	21.0	[33]
Perenniporia fraxinea	VLPITKELLPPEFFVA	6.3	42.0	[35]
Flammulina velutipes (FVP-1)	LTYRVIPITKQAVTEG	12.5	37.0	[34]
Paecilomyces tenuipes (PTEFP)	AQNIGAVVNLSPPKQ	0	14.0	[7]

Fibrinolytic Activity

To investigate the fibrin hydrolyzing capacity of herinase, both plasminogen-free and plasminogen-rich fibrin plate method was used. As illustrated in Fig. 3, application of herinase produced clear zone in plasminogen-free fibrin plate and the size of clear zone was larger than plasmin. Interestingly, the clear area due to herinase was enhanced in the presence of plasminogen (data not shown). These results suggest that herinase has dual property, which can hydrolyze the fibrin directly as well as activate the plasminogen. In the fibrin plates, the edge of the clear zone by herinase was different than plasmin, which may be due to the difference in the cleavage patterns of fibrin chain.

To further analyze its fibrinolytic activity, partially cross-linked fibrin clot prepared with human thrombin was incubated with herinase for different times and the hydrolyzed products were examined by SDS–PAGE. Plasmin was used as control. As shown in Fig. 4, herinase rapidly cleaved the α chain of fibrin, followed by hydrolysis of the γ–γ chains. However, the β chain of fibrin remained intact during the incubation period (3 h). The cleavage pattern of fibrin clot due to herinase was different from the hydrolysis induced by plasmin.

Table 3 Amidolytic activity of herinase towards chromogenic substrates

Chromogenic substrate	Amino acid sequence	Characteristics	Activity (μmol/min/mg)
S-2238	H-D-Phe-Pip-Arg-pNA	For thrombin	7.5±0.5
S-2765	Z-D-Arg-Gly-Arg-pNA·2HCl	For factor Xa	8.0±0.4
S-2251	H-D-Val-Leu-Lys-pNA	For plasmin	11.2±0.4
S-2444	pyroGlu-Gly-Arg-pNA	For urokinase	4.7±0.0
S-2288	H-D-Ile-Pro-Arg-PNA	For t-PA	16.7±0.5

Each value represents the mean±SD for three determinations

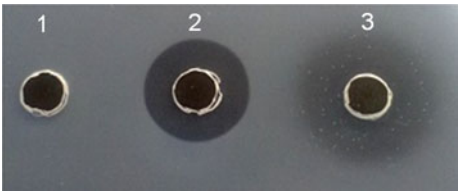

Fig. 3 Fibrinolytic activity of herinase by fibrin plate method. Fibrin plate was prepared by mixing agarose, human fibrinogen, and thrombin. Ten microliters of herinase (1.0 μg) was applied to the wells in fibrin plate and incubated for 12 h at 37 °C. Plasmin (1.0 μg) was used as standard. *1* DW, *2* plasmin, *3* herinase

Fibrinogenolytic Activity

To address its ability to hydrolyze human fibrinogen, herinase was incubated with human fibrinogen and analyzed by SDS–PAGE. Fibrinogen degradation pattern obtained showed that herinase rapidly hydrolyzed Aα chains of fibrinogen followed by γ chains. However, similar to fibrin, the Bβ chains of fibrinogen was also resistant to hydrolysis (Fig. 5) during the incubation time point. The fibrinogenolytic patterns suggest that herinase has some properties similar to that of α-fibrinogenase, which preferentially hydrolyzed the Aα chain of fibrinogen rather than the Bβ and γ chains [26].

Effects of pH and Temperature on Fibrinolytic Activity of Herinase

The effects of pH on fibrinolytic activity are shown in Fig. 6a. Herinase was found to be active between the pH ranges 4.0 and 9.0, with maximum activity at pH 7.0. The enzyme activity was decreased in highly acidic and basic environment. However, the activity of enzyme was found stable in pH ranges of 5.5–7.0. The optimum temperature of herinase is shown in Fig. 6b. The optimum temperature for herinase activity was 30 °C. The enzyme was relatively stable below 40 °C. However, above 45 °C the activity of enzyme was sharply reduced.

Effect of Metal Ions and Protease Inhibitors on the Fibrinolytic Activity

The influence of metal ions on fibrinolytic activity of herinase was investigated in the presence of different metal ions for 1 h at 37 °C. The metal ions such as Ca^{2+}, Mg^{2+}, and Mn^{2+} were found to increase the enzyme activity significantly (Fig. 7a). However, the addition of Cu^{2+}, Fe^{2+}, and Zn^{2+} decreased the activity remarkably.

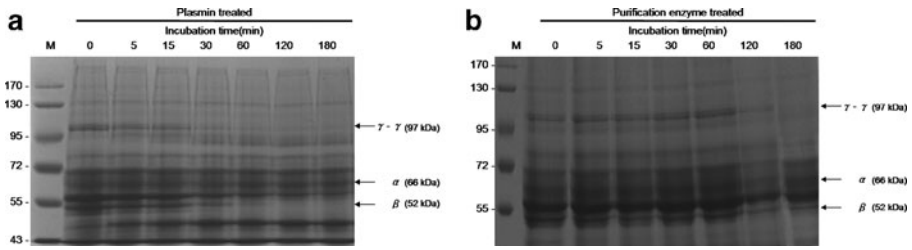

Fig. 4 Degradation of fibrin clot by plasmin (**a**) and herinase (**b**). Fibrin clot was prepared with human fibrinogen and thrombin in 50 mM Tris–HCl (pH 7.4) containing 0.1 M NaCl and incubated with 1 μg of enzyme for 0–180 min. Degradation products were analyzed by SDS–PAGE. *Lane M* molecular weight marker

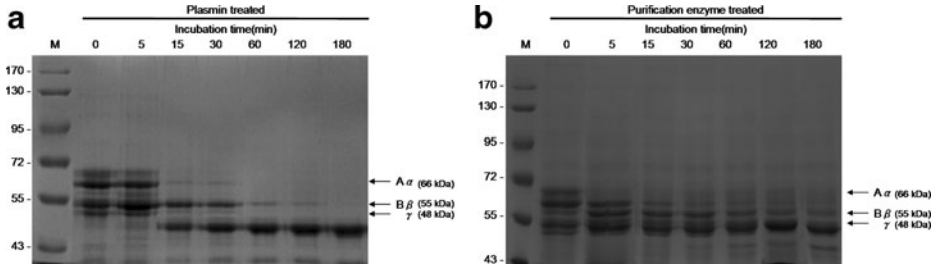

Fig. 5 Degradation of human fibrinogen by plasmin (**a**) and herinase (**b**). Human fibrinogen (1 %) was incubated with 1 μg of herinase or plasmin in 50 mM Tris–HCl (pH 7.4) containing 0.1 M NaCl at 37 °C for 0–180 min. Then after, the reaction mixtures were analyzed by SDS–PAGE. *Lane M* molecular weight marker

To determine the class of herinase, the inhibition assay was performed with different class of protease inhibitors such as PMSF, TLCK, TPCK, aprotinin, EDTA, and EGTA. As shown in Fig.7b, the relative activity of herinase was highly suppressed after the addition of metalloprotease inhibitors such as EDTA and EGTA. On the other hand, the activity was not affected by other inhibitors including PMSF and TPCK. These results indicate that herinase is a metalloprotease.

Discussion

Progresses in understanding the mechanism of fibrin clot dissolution open new avenues for the development of potential agents in fibrinolytic therapy. Although current clinically used thrombolytic drugs are mostly plasminogen activators and t-PA is still regarded as a gold standard in this therapy [26], these agents pose unavoidable risk of bleeding. Therefore, pharmacological and clinical studies are now focused on safe and effective thrombolytic treatment strategies. Recently, direct acting fibrinolytic agents, which have shown impressive thrombolytic efficacy and safety from bleeding, and may represent viable alternative to t-PA [27], have received abundant attention in the scientific community. The exogenous proteases with dual activity could be interesting and a potential candidate for thrombolytic

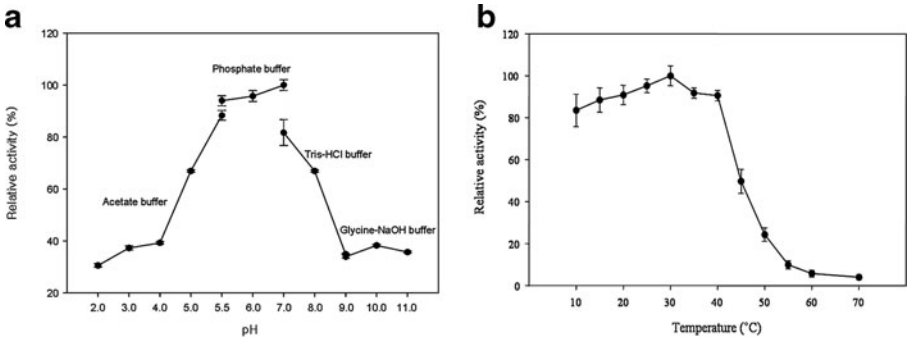

Fig. 6 Effect of pH (**a**) and temperature (**b**) on fibrinolytic activity of herinase. Herinase (1 μg) was added to 90 μl of 50 mM glycine–HCl, citric–NaOH, Tris–HCl, and glycine–NaOH buffer systems and incubated for 1 h. Relative activity was expressed as a percentage of the maximum enzyme activity under the assay conditions. The optimal temperature was determined after the incubation of 1.0 μg of herinase at different temperatures (10–70 °C) for 1 h. Each value represents the mean±SD for three determinations

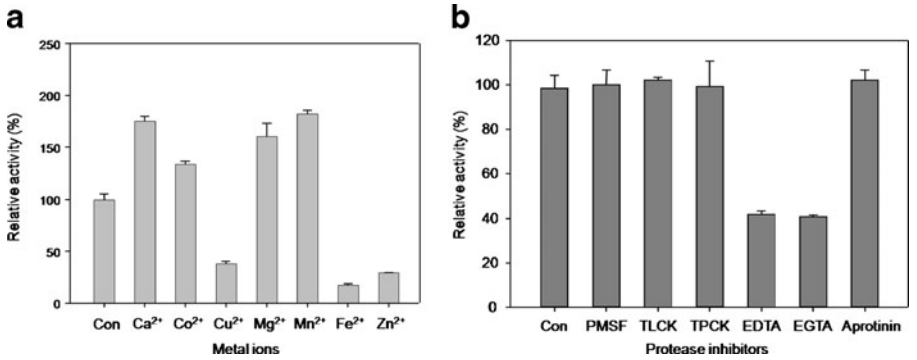

Fig. 7 Effects of metal ions (**a**) and protease inhibitors (**b**) on the fibrinolytic activity of herinase. The purified enzyme (1.0 μg) was mixed with metal ions or inhibitors in 50 mM Tris–HCl for 1 h at 37 °C. Each enzyme solution (10 μl) was then incubated with fibrin clot prepared from human fibrinogen and thrombin as described in "Materials and Methods" at 37 °C for 10 min and the enzyme activity was measured by a microplate reader. The level of inhibition was expressed as a percentage of the relative activity to the control activity (without metal ion or inhibitor). Each value represents the mean±SD for three determinations

therapy. However, the existence of such bifunctional agents that could activate plasminogen as well as degrade the fibrin clot directly had not been reported previously from mushroom. In the present study, we describe the purification and biochemical characterization of a 51-kDa bi-functional fibrinolytic metalloprotease designated as "herinase" from the fruiting bodies of *H. erinaceum*.

One distinguishing feature of herinase is that it can hydrolyze the fibrin independently as plasmin. Fibrin is a favorable substrate for plasmin and fibrin zymography has been suggested to be a reliable technique to demonstrate plasmin-like enzymes [21, 28]. We have therefore applied fibrin zymography to identify protease activity in SDS–PAGE. As shown in Fig. 2b, herinase produced a clear and sharp band of 51 kDa on fibrin zymogram, indicating that herinase has plasmin-like activity. Moreover, we found that herinase induced fibrinolytic activity on both plasminogen-free and plasminogen-rich fibrin plates. These results indicate that two mechanisms could be involved in herinase-mediated fibrinolysis: (1) direct acting and (2) via the activation of plasminogen. Only a few fibrinolytic enzymes such as lumbrokinase and eupolytin 1 have been reported to possess such bi-functional activity [29, 30]. Bi-functionality is the unique feature of herinase, which makes it superior than other fibrinolytic enzymes. Moreover, to understand the substrate specificity of herinase, the hydrolytic activities toward various peptide substrates were measured. The results from chromogenic substrates revealed higher activity with S-2288, substrate for t-PA, followed by S-2251, substrate for plasmin (Table 3), indicating that herinase contains both t-PA- and plasmin-like activities, in general agreement with the trends discussed above.

Another important feature of herinase is its ability to hydrolyze fibrin and fibrinogen. The fibrin(ogen)olytic pattern by herinase differs from other fibrinolytic enzymes because it appeared to target on Aα chain of fibrinogen, one of the most important proteins for coagulation in plasma, which is quickly digested by hydrolyzing peptide bonds different from those hydrolyzed by plasmin (Fig. 5). This pattern of cleavage is, however, similar to that of α-fibrinogenase from different sources such as snake venom [26, 31], *Lampetra japonica* [19], *Codium divaricatum* [24], and *Paecilomyces tenuipes* [7]. Interestingly, unlike many fibrinolytic enzymes from above mentioned and other sources, herinase could also cleave the γ chain of fibrinogen. Since the γ chain does not appear to be a specific target

of fibrinolytic enzymes from snake venom [26], a metalloprotease from mushroom, for example, *Tricholoma saponaceum*, could cleave both the Aα and Bβ chains but has no activity toward the γ chain [32]. Moreover, herinase also exhibited a different pattern of specificity with respect to the cleavage of fibrin than other known fibrinolytic enzymes. Herinase was capable of digesting the α and γ–γ chain of fibrin but did not show activity toward the β chain, suggesting that herinase could be different from other proteases.

Additionally, the N-terminal sequence analysis revealed that herinase is a novel protease. Herinase shares a high sequence identity with the metallopeptidase. Interestingly, herinase exhibited a weak or no homology in N-terminal amino acid sequence with other known fibrinolytic enzymes. Moreover, the uniqueness of the herinase was observed in the dependence of the fibrinolytic activity on protease inhibitors and various metal ions. Protease assay with inhibitors and metal ions revealed that some features of herinase were different from those of some fungal fibrinolytic metalloproteases previously reported from our laboratory [33–35], and from other mushrooms [5] and food-grade microorganisms [36]. Inhibition assay showed that the activity of herinase was strongly inhibited by EDTA and EGTA. Sequence comparison and inhibition of herinase by these chelating agents established that it is a metalloprotease. It has been reported that the fibrinolytic serine proteinases and coagulation of blood are usually targeted by serpins (serine protease inhibitors) [37]. Therefore, the specific advantage of herinase over fibrinolytic serine proteases is that it will not be inhibited by the blood serpins. Furthermore, similar to other direct-acting metalloproteases, the proteolytic activity of herinase was also activated by calcium and magnesium ion. These ions are suggested to be important for structural stabilization of metalloprotease [38, 39].

In conclusion, we report the isolation and biochemical properties of the fibrin(ogen)olytic metalloprotease from medicinal and edible mushroom *H. erinaceum*. Because of its ability to promote fibrin(ogen)olytic activity directly as well as through the activation of plasminogen, herinase seems to be a promising potential candidate for further study in thrombolytic therapy.

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Conflict of Interest None

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