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Purification and Biochemical Characterization of a 17 kDa Fibrinolytic Enzyme from *Schizophyllum commune*

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A fibrinolytic enzyme of the mushroom, *Schizophyllum commune* was purified with chromatographic methods, including a DEAE-Sephadex A-50 ion-exchange column and gel filtrations with Sephadex G-75 and Sephadex G-50 columns. The analysis of fibrin-zymography and SDS-PAGE showed that the enzyme was a monomeric subunit that was estimated to be approximately 17 kDa in size. The fibrinolytic activity of the enzyme in plasminogen-rich and plasminogen-free fibrin plates was 1.25 and 0.44 U/ml, respectively. The N-terminal amino acid sequence of the purified enzyme was identified as HYNIXNSWSSFID, which was highly distinguished from known fibrinolytic enzymes. The relative activity of the purified enzyme with an addition of 5 mM EDTA, Phosphoramidon, and Bestatin was about 76, 64, and 52%, respectively, indicating that it is a metalloprotease. The optimum temperature for the purified enzyme was approximately 45°C, and over 87% of the enzymatic activity was maintained as a stable state in a pH range from 4.0 to 6.0. Therefore, our results suggest that the potential thrombolytic agent from *S. commune* is a unique type of fibrinolytic enzyme.

***Keywords*:** fibrinolytic enzyme, fibrin-zymography, N-terminal amino acid sequence, mushroom, *S. commune*

Cardiovascular diseases involving the blood circulation system usually result from fibrin clots adhering to the walls of blood vessels, leading to thrombosis (Bode *et al*., 1996; Dobrovolsky and Titaeva, 2002). Fibrinolysis studies on the treatment of such cardiovascular diseases have progressed rapidly in recent history. Typically, fibrinolytic enzymes, such as tissue plasminogen activator (*t*-PA) and urokinase (*u*-PA, EC 3.4.21.31), have been used as thrombolytic agents (Husain *et al*., 1983; Blann *et al*., 2002). The fibrinolytic enzymes produced by the microbes, including streptokinase and staphylokinase, have been widely investigated (Medved *et al*., 1966; Sumi *et al*., 1987; Lijnen *et al*., 1991; Kim *et al*., 1997, 2010a, 2010b; Lee *et al*., 1999; Jeong *et al*., 2001, 2004a, 2004b), however, they are not commonly used because of side effects that include hemorrhaging, allergic reactions, and a relapse (Bode *et al*., 1996; Blann *et al*., 2002).

Historically, fermented soybean foods including natto (Nakamura *et al*., 1992) and Chungkook-Jang (Kim *et al*., 1996) have been commonly used as thrombolytic agents. A wide variety of bioactive compounds from mushrooms have also been addressed as potential thrombolytic agents (Lo *et al*., 2006). Recently, fibrinolytic activities in several mushrooms, including *Tricholoma saponaceum*, *Armillariella mellea*, and *Cordyceps militaris*, have been reported (Kim and Kim, 2001; Lee *et al*., 2005; Kim *et al*., 2006). The macrofungal fibrinolytic enzymes produced by a submerged mycelial culture can be used in many medicinal applications (Wasser, 2002; Cui and Chisti, 2003).

Few studies reporting on the proteolytic enzymes from *Shizophyllum commune*, a common mushroom that occurs on every continent except Antarctica, have been conducted. Recently, hemolysin from the fruiting bodies of *S. commune* was purified with affinity chromatography and gel filtration using a Superdex 75 column (Han *et al*., 2010). However, no efforts have been made to produce extracellular metabolites with a submerged mycelial culture of a mushroom, *S. commune*. In order to identify an extracellular fibrinolytic enzyme from the mushroom-forming basidiomycete, we created submerged mycelial cultures and purified the enzyme using ion-exchange chromatography and gel filtration. In addition, we biochemically characterized the purified enzyme.

Materials and Methods

Fungal strain and submerged mycelial culture

The mycelium of the fungal strain, *S. commune* KCTC 6482 was purchased from the Korean Agricultural Culture Collection (KACC), Suwon, Korea. A culture of the strain was maintained on malt extract agar slants (2% malt extract, 1.5% agar) stored at 4°C and subcultured every 2 weeks. The seed culture was grown in a 250 ml flask containing 50 ml of a mushroom complete medium (MCM): (g/L) 20 glucose, 2.0 meat peptone, 2.0 yeast extract, 0.46 KH_2PO_4, 1.0 K_2HPO_4, and 0.5 $MgSO_4 \cdot 7H_2O$ at 25°C on a rotary shaker incubator at 250 rpm for 7 days. The MCM was used throughout the study for the submerged mycelial culture of the *S. commune* strain.

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Enzyme purification

To prepare the crude enzyme, the cultured mycelium of the *S. commune* strain was removed by centrifuging at 9,000 rpm for 20 min, and ammonium sulfate was slowly added to the supernatant up to 80% saturation. To harvest extracellular proteins, the mixture of supernatant and ammonium sulfate was allowed to stand at 4°C for 12 h. The resulting precipitate was dissolved in a 20 mM sodium phosphate buffer (pH 7.0) and centrifuged at 13,000 rpm for 5 min to remove the insoluble debris. The crude extracts were prepared by dialyzing in 5 L of the buffer three times, centrifuging at 13,000 rpm for 20 min, and dissolving in 3 ml of the buffer.

All purification steps were performed at 4°C. Protein concentration was measured according to the method used by Bradford (1976). The enzyme was first purified by chromatography using a DEAE-Sephadex A-50 column. The enzyme was eluted by a linear gradient of 0.1 to 0.5 M sodium chloride. The active fractions showing fibrinolytic activity, which were identified with fibrin plate methods (Astrup and Müllertz, 1952), were pooled, precipitated with ammonium sulfate (80% saturation), dialyzed using the buffer, and loaded onto a Sephadex G-75 column previously equilibrated in the buffer. An eluted protein solution was pooled, dialyzed, and loaded on a Sephadex G-50 column. The active fractions obtained from the Sephadex G-75 column were then pooled, precipitated with ammonium sulfate (80% saturation), dialyzed using the buffer, and lyophilized. A sodium phosphate buffer (pH 7.0) was used as an elution buffer and flow rate was 0.5 ml/min.

SDS-PAGE and fibrin-zymography analysis

To determine the molecular mass of the protein purified by gel filtration on the Sephadex G-50, SDS-PAGE was performed according to the method described by Laemmli (1970), using a 10% gradient polyacrylamide gel and 5% stacking at 4°C. The fibrin-zymography analysis of the protein was carried out according to the method described by Markus *et al*. (1984), except a 0.12% fibrinogen solution rather than distilled water was used in the preparation of separating gels. The activity band was recognizable as a colorless area in the gel depleted of fibrin against a blue background.

Estimation of fibrinolytic activity

Fibrinolytic activity was determined with fibrin plate methods (Astrup and Müllertz, 1952), using plasminogen-free and plasminogen-rich plates. Plasminogen-free fibrin plates were supplemented with fibrinogen solution [2.5 ml of 1.2% (w/v) human fibrinogen (Sigma, USA) in 0.1 M sodium phosphate buffer, pH 7.4], 10 U of thrombin solution (Sigma), and 1% agarose. Then, plasminogen-free fibrin plates were prepared by heating at 80°C for 30 min. Plasminogen-rich fibrin plates were supplemented with 1.5% fibrinogen, 5 U of plasmonogen, and 1% agarose.

A hole (5 mm in diameter) was punched in the fibrin plate for sample application and a sterilized paper disc was placed on the plate. To observe fibrinolytic activity, 100 μl of the purified protein solution was dropped onto the disc, and the plate was incubated at 37°C for 18 h. The activity of the fibrinolytic enzyme was determined by measuring a halo zone on the fibrin plate and plotting it to a standard curve, which was drawn by estimating the lysis area shown on a plasminogen-rich fibrin plate given a standard amount of a protein, plasmin.

Estimation of amidolytic activity for synthetic substrates

Amidolytic activity was measured spectrophotometrically with chromogenic substrates including plasmin, thrombin, and kallikrein. The reaction mixture (1 ml) contained 20 μl of enzyme solution, 5×10^{-4} M chromogenic substrate, and 0.1 M sodium phosphate buffer (pH 7.4). After incubation for 5 min at 37°C, the amount of liberated *p*-nitroaniline was determined from spectrophotometric absorption at 405 nm.

Effect of metal ions and protease inhibitors

The effect of metal ions, $ZnCl_2$, $HgCl_2$, $CoCl_2$, $CuSO_4$, $FeSO_4$, $MgCl_2$, and $MnSO_4$ was observed. The influence of various protease inhibitors, EDTA, Phosphoramidon, Bestatin, Fefabloc, PMSF, Leucopeptin, E-64, Chymostatin, and Antipain on the fibrinolytic activity was analyzed. The enzymatic activity was measured spectrophotometrically by supplementation with a chromogenic substrate during the enzyme reaction. The reaction mixture (1 ml) contained 20 μl of enzyme solution, 5×10^{-4} M chromogenic substrate, and 0.1 M sodium phosphate buffer (pH 7.4). After incubation for 5 min at 37°C, the amount of liberated *p*-nitroaniline was determined from spectrophotometric absorption at 405 nm. One unit of amidolytic activity was expressed as μmole of substrate hydrolyzed per min per mg of protein. The concentrations of all protease inhibitors and metal ions in the reactions were 5 mM.

Effects of temperature and pH on the fibrinolytic activity

The optimum temperature for the fibrinolytic activity was determined by keeping the purified enzyme in a 10 mM phosphate buffer (pH 7.4) for 30 min at various temperatures (20, 25, 30, 35, 40, 45, 50, 55, and 60°C). The optimal pH for the fibrinolytic activity was determined in a pH range of 3.0 to 10.0, using the following buffer systems: 0.05 M citrate buffer (pH 4.0), 0.05 M sodium phosphate (pH 5.0-7.0), 0.05 M Tris-HCl (pH 8.0-9.0), and 0.05 M glycine-NaOH (pH 10.0).

N-terminal amino acid sequencing

The fibrinolytic protein purified on a SDS-PAGE gel was electroblotted to a polyvinylidene difluoride membrane (Bio-Rad, USA) and stained with Coomassie Brilliant Blue R-250 (Sigma). The stained protein band was excised, and the amino acids of the N-terminal sequence were determined with the automated Edman method using a gas-phase protein sequencer (Model 476A, Applied Biosystems, USA). The analysis of the N-terminal amino acid sequence was performed by the BLAST program of the NCBI bank (http://www.ncbi.nlm.nih.org).

Results and Discussion

Purification of a fibrinolytic enzyme, and analysis of SDS-PAGE and fibrin-zymography

The fibrinolytic enzyme was purified with the steps described above, including ion exchange chromatography with a DEAE Sephadex A-50 column and gel filtration with Sephadex G-75 and Sephadex G-50 columns (Table 1). In comparison with the culture supernatant, the specific activity of the enzyme after purification was increased 67-times, with a recovery of 2.8%. A monomeric subunit showing high fibrinolytic activity was shown on the SDS-PAGE and fibrin-zymography gels. The single polypeptide was estimated to be approximately 17 kDa in size, and was designated as Mushrokinase (MsK) (Fig. 1). The proteolytic activity of the enzyme was observed with a fibrin plate assay for 4 h (Fig. 2). The fibrinolytic activity of the purified enzyme, MsK, in plasminogen-rich and plasminogen-free fibrin plates was 1.25 and 0.44 U/ml, respectively (Fig. 2C). These results are particularly interesting since MsK is

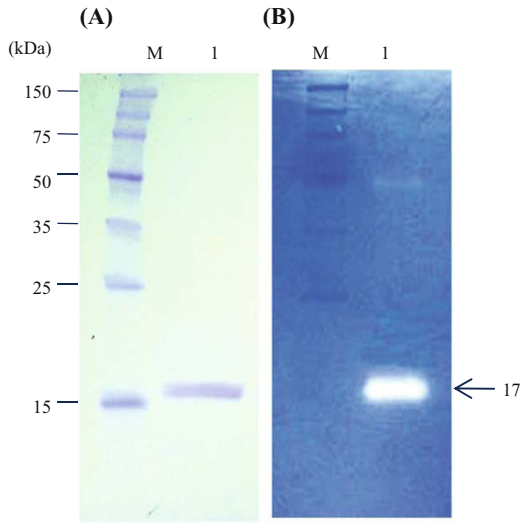

Fig. 1. SDS-PAGE (A) and fibrin-zymography (B) of the fibrinolytic enzyme (MsK) purified from *S. commune* KCTC 6482. (A) Lanes: M, protein molecular mass markers; 1, purified enzyme from Sephadex G-50 gel filtration. (B) Lanes: M, protein molecular mass markers; 1, purified enzyme from Sephadex G-75 gel filtration. The arrow indicates the fibrinolytic enzyme purified from *S. commune* KCTC 6482.

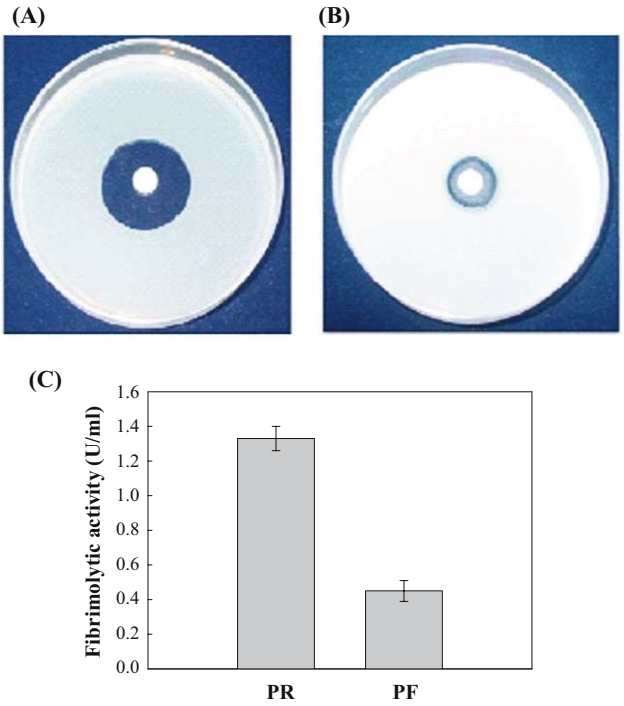

Fig. 2. Fibrinolytic activity of the enzyme, MsK, in plasminogen-rich fibrin plate (A), plasminogen-free fibrin plate (B), and estimation of fibrinolytic activity (C). The results in (C) represent the Mean±SD for three independent experiments. PR and PF represent plasminogen-rich fibrin plate and plasminogen-free fibrin plate, respectively. One unit in Fig. 2C is expressed as μmole of plasmin produced per min by 1 mg of protein.

capable of degrading fibrin directly (*direct type*) and can also degrade it by forming plasmin from plasminogen (*plasminogen activator type*). Only a few fibrinolytic enzymes have been known to exert dual functions of plasminogen activator and direct type (Mihara *et al*., 1991).

We next analyzed the amidolytic activity of MsK with commercial substrates (Table 2). Under the presence of the synthetic substrate for plasmin, the amidolytic activity of MsK was 6.9- and 7.5-times higher than that of the known fibrinolytic enzymes, subtilisin Carlsberg (Smith *et al*., 1968) and CK (Kim *et al*., 1996), respectively. In the presence of the synthetic substrate for thrombin, the amidolytic activity of MsK showed 5.3- and 11.9-times higher than that of Carlberg and CK, respectively, and when using the synthetic substrate for kallikrein, the amidolytic activity of the protein was 5.8- and 8.1-times higher than that of subtilisin Carlsberg and CK, respectively.

Effects of protease inhibitors and metal ions

The effects of various protease inhibitors on the fibrinolytic activity of MsK were observed (Table 3). The relative activity of MsK after the addition of metalloprotease inhibitors EDTA, phosphoramidon, and Bestatin was about 24-, 36-, and 48%, respectively, suggesting that MsK is a metalloprotease. However, other inhibitors, including Fefabloc, PMSF, Leupeptin, E-64, Chymostatin, and Antipain, had no significant effect on the fibrinolytic activity of MsK.

The influence of various metal ions on the fibrinolytic activity was also observed (Table 3). The addition of Zn^{2+} to the reaction increased the enzymatic activity by 63%, while the addition of Hg^{2+} decreased the activity by 39%. None of the other metal ions, including Co^{2+}, Cu^{2+}, Fe^{2+}, Mg^{2+}, and Mn^{2+}, had a significant effect on the fibrinolytic activity of MsK.

Table 1. Purification of the 17 kDa fibrinolytic enzyme (MsK) from *S. commune* KCTC 6482

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg protein)	Purification (Fold)	Yield (%)
Culture supernatant	8,026	4,680	0.6	1	100
80% Ammonium sulfate Precipitation	215.8	1,106	5.1	8.5	23.5
DEAE A-50	14.3	358	25.0	41.7	7.6
Sephadex G-75	5.3	194.3	36.7	61.1	4.1
Sephadex G-50	3.2	129	40.3	67.2	2.8

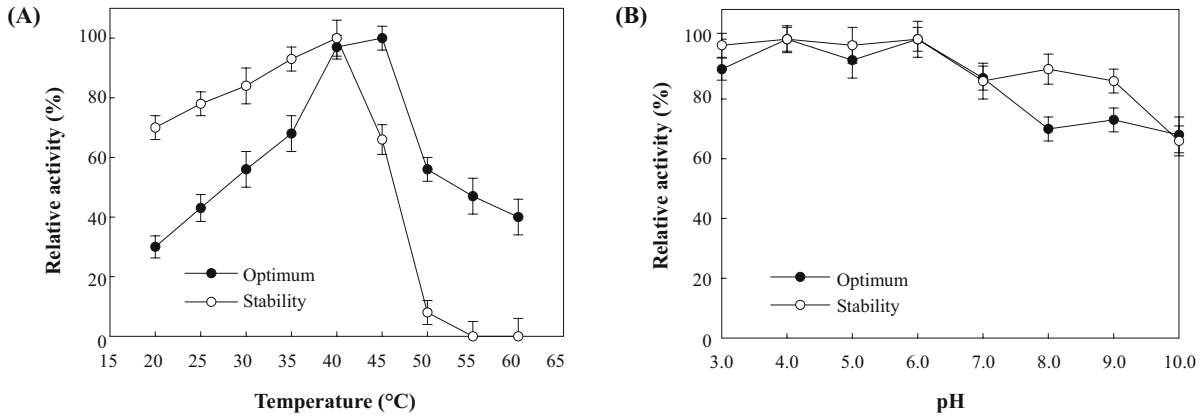

Fig. 3. Optimum temperature (A) and pH (B) of the fibrinolytic enzyme (MsK) purified from *S. commune*. Results represent the mean±SD for three independent experiments.

Effects of temperature and pH on fibrinolytic activity

The fibrinolytic activity of MsK measured at temperatures between 20 and 60°C showed that activity was the highest at 40°C and decreased significantly at temperatures over 50°C (Fig. 3A). More than 92% of fibrinolytic activity was maintained at 45°C for 30 min. The optimum pH range of MsK was between 4.0 and 6.0, with high stability for all pH's within that range (Fig. 3B). This characteristic, high stability over a broad pH range, is shared by the enzymes of several basidiomycetes (Wannet *et al*., 2000; Tlecuitl-Beristain *et al*., 2008).

N-terminal amino acid sequencing

The first 14 amino acid residues of the MsK N-terminal sequence were HYNIXNSSWSSFID (Table 4). This N-terminal amino acid sequence is distinguished from that of fibrinolytic enzymes from other fungal strains, including TSMEP1 from *Tricholoma saponaceum* (Kim and Kim, 2001), the 18.5 kDa enzyme from *Armillariella mellea* (Kim and Kim, 1999), AMMP1 from *A. mellea* (Lee *et al*., 2005), the 52 kDa enzyme from *Cordyceps militaris* (Kim *et al*., 2006), FVP-I from *Flammulina velutipes* (Park *et al*., 2007), and PoFE from *Pleurotus ostreatus* (Shen *et al*., 2007). None of the sequences of other known fibrinolytic enzymes were significantly homologous to MsK.

Table 2. Amidolytic activity of the 17 kDa fibrinolytic enzyme (MsK), subtilisin Carlsberg, and CK for the synthetic substrates

Substrate	Enzyme activity[a]		
	MsK	Carlsberg	CK
H-D-Val-Leu-Lys-pNA[b]	2979 (100)	434 (100)	398 (100)
H-D-Phe-Pip-Arg-pNA[c]	249 (8.3)	47 (10.8)	21 (5.3)
H-D-Val-Leu-Arg-pNA[d]	138 (4.6)	24 (5.5)	17 (4.3)

The values in parentheses are percentages calculated on the basis of enzyme activity to H-D-Val-Leu-Lys-pNA. Each value is the mean of three determinations.

[a] Enzyme activity was expressed as nmole of substrate hydrolyzed per minute by 1 mg of protein

[b] H-D-Val-Leu-Lys-pNA (S-2251): synthetic substrate for plasmin

[c] H-D-Phe-Pip-Arg-pNA (S-2238): synthetic substrate for thrombin

[d] H-D-Val-Leu-Arg-pNA (S-2266): synthetic substrate for kallikrein

Recently, a hemolytic enzyme, Shizolysin was characterized in the fruiting bodies of *S. commune* (Han *et al*., 2010). In this study, we purified and characterized a new fibrinolytic enzyme, MsK, from the mycelia-cultured products of *S. commune*. We distinguished MsK from other fibrinolytic enzymes with respect to N-terminal amino acid sequence. The enzyme exerted a dual function in fibrin degradation, acting as a plasminogen activator and direct type. Our research suggests that MsK is a metalloprotease that conserves Zn^{2+} and/or Co^{2+} in its active center. This study exposes an extracellular fibrinolytic enzyme, a potential thrombolytic agent, from the submerged mycelial culture of the mushroom.

Table 3. Effect of protease inhibitors and metal ions on fibrinolytic activity of the fibrinolytic enzyme, MsK

Inhibitor or metal ion	Relative activity (%)[a]
None	100
Zn^{2+}	166±2.9
Hg^{2+}	58±2.6
Co^{2+}	154±3.7
Cu^{2+}	88±2.1
Fe^{2+}	82±2.2
Mg^{2+}	79±2.7
Mn^{2+}	72±3.6
EDTA	23±1.2
Phosphoramidon	34±1.6
Bestatin	46±1.8
Fefabloc	92±4.2
PMSF	95±2.8
Leucopeptin	91±1.8
E-64	93±2.2
Chymostatin	94±3.1
Antipain	89±2.1

[a] Activity relative to that of the fibrinolytic enzyme, MsK, from *S. commune* KCTC 6482, which was considered to be 100%. The values shown are the averages of at least three independent experiments. The concentration of all protein inhibitors and metal ions was 5 mM.

Table 4. Comparison of N-terminal amino acid sequence between the fibrinolytic enzyme (MsK) purified from *S. commune* and the known protease from mushrooms

Fibrinolytic enzyme	Fungal strains	N-terminal sequence	Reference
MsK	*Schizophyllum commune*	HYNIXNSSWSSFID	This study
18.5 kDa enzyme	*A. mellea*	XXYNGXTXSRQTTL	Kim and Kim (1999)
TSMEP1	*Tricholoma saponaceum*	ALYVGXSPXQQSLL	Kim and Kim (2001)
AMMP1	*A. mellea*	MFSLSSRFFLYTLC	Lee *et al*. (2005)
52 kDa enzyme	*Cordyceps militaris*	ALTTQSNVTHGLAT	Kim *et al*. (2006)
FVP-I	*Flammulina velutipes*	LTYRVPITKQAVTE	Park *et al*. (2007)
PoFE	*Pleurotus ostreatus*	ALRKGGAAALNIYS	Shen *et al*. (2007)

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