The Journal of Microbiology, December 2006, p. 622-631
Vol. 44, No. 6

A Fibrinolytic Enzyme from the Medicinal Mushroom *Cordyceps militaris*

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(Received August 22, 2006 / Accepted November 22, 2006)

In this study we purified a fibrinolytic enzyme from *Cordyceps militaris* using a combination of ion-exchange chromatography on a DEAE Sephadex A-50 column, gel filtration chromatography on a Sephadex G-75 column, and FPLC on a HiLoad 16/60 Superdex 75 column. This purification protocol resulted in a 191.8-fold purification of the enzyme and a final yield of 12.9%. The molecular mass of the purified enzyme was estimated to be 52 kDa by SDS-PAGE, fibrin-zymography, and gel filtration chromatography. The first 19 amino acid residues of the N-terminal sequence were ALTTQSNV THGLATISLRQ, which is similar to the subtilisin-like serine protease PR1J from *Metarhizium anisopliae var. anisopliase*. This enzyme is a neutral protease with an optimal reaction pH and temperature of 7.4 and 37°C, respectively. Results for the fibrinolysis pattern showed that the enzyme rapidly hydrolyzed the fibrin α-chain followed by the γ-γ chains. It also hydrolyzed the β-chain, but more slowly. The Aα, Bβ, and γ chains of fibrinogen were also cleaved very rapidly. We found that enzyme activity was inhibited by Cu^{2+} and Co^{2+}, but enhanced by the additions of Ca^{2+} and Mg^{2+} ions. Furthermore, fibrinolytic enzyme activity was potently inhibited by PMSF and APMSF. This enzyme exhibited a high specificity for the chymotrypsin substrate S-2586 indicating it's a chymotrypsin-like serine protease. The data we present suggest that the fibrinolytic enzyme derived from the edible and medicinal mushroom *Cordyceps militaris* has fibrin binding activity, which allows for the local activation of the fibrin degradation pathway.

Keywords: *Cordyceps militaris*, Fibrinolytic enzyme, Serine protease, Subtilisin, Fibrinolysis, Thrombus

Hemostasis is a tightly regulated process for keeping an optimal balance between coagulation and anticoagulation. The maintenance of this process is essential for the prevention of bleeding and thrombosis, which occurs by the sequential and short-lived activation of a coagulation cascade series of enzymes. This process ultimately results in the production of an insoluble fibrin clot. Fibrin is the primary protein component of blood clots, which are formed from fibrinogen by thrombin (Voet and Voet, 1990). The insoluble fibrin fiber is hydrolyzed into fibrin degradation products by plasmin, which is generated from plasminogen by plasminogen activators such as tissue plasminogen activator (t-PA), vascular plasminogen activator, blood plasminogen activator, urokinase, Hageman factor, and streptokinase-plasminogen complex (Collen, 1980; Collen and Lijnen, 1991). Fibrin clot formation and fibrinolysis are normally well balanced in biological systems. However, when fibrin is not hydrolyzed, due to some disorder, thromboses can occur. Myocardial infarction is the most common form of such thromboses.

The fibrinolytic agents available today for clinical use are mostly plasminogen activators such as the tissue-type plasminogen activator, the urokinase-type plasminogen activator, and the bacterial plasminogen activator streptokinase. Despite their widespread use

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all these agents have undesired side effects, exhibit a low specificity for fibrin, and are relatively expensive. Therefore, the search for other fibrinolytic enzymes from various sources continues. Recently, potent fibrinolytic enzymes have been discovered in snake venom, earthworm secretions, dung beetles, food-grade microorganisms, marine creatures, herbal medicines, and fermented food products like Japanese *Natto* and Korean *Chungkook-Jang* soy sauce (Nikai *et al.*, 1984; Sumi *et al.*, 1987; Mihara *et al.*, 1991; Sumi *et al.*, 1992; Kim *et al.*, 1996; Chang *et al.*, 2000; Choi and Sa, 2000; Jeong *et al.*, 2001; Ahn *et al.*, 2004).

In recent years, mushrooms have become an attractive source of physiologically active compounds (Wasser and Weis, 1999; Zaidman *et al.*, 2005). They are typically used as food and food flavoring substance, and also as traditional oriental medicines. Their extracts have been reported to exert hematological, antiviral, antitumorigenic, hypotensive, and hepatoprotective effects (Chang and Miles, 1989; Hobbs, 1995; Chang, 1996). Mushrooms also constitute an important source of thrombolytic agents. Korean traditional anecdotes suggest that mushrooms can be, and have been, used in the treatment and prevention of thrombosis (Kim and Kim, 1995). Some reports have described the fibrinolytic activity of certain edible mushrooms, including *Flammulina velutipes* (Choi *et al.*, 1999), *Pleurotus ostreatus* (Dohmae *et al.*, 1995; Shin and Choi, 1999; Joh *et al.*, 2004), *Grifola frondosa* (Datta *et al.*, 1995; Nonaka *et al.*, 1995), *Tricholoma saponaceum* (Kim and Kim, 2001), and *Armillaria mellea* (Kim *et al.*, 1998; Lee *et al.*, 2005). *Cordyceps militaris* is a popular Chinese medicinal mushroom, and an entomogenous fungal species important for the biocontrol of pine moth populations (Alicja, 1998). Entomogenous fungal species invade and proliferate within insect larvae causing a systematic infection that eventually kills the host (Clarkson and Charnley, 1996). *Cordyceps militaris* has received extensive attention for possible medical applications due to its various physiological activities (Wu *et al.*, 2000; Ng and Wang, 2005). Cordycepin (3′-deoxyadenosine), one of its major bioactive secondary metabolites, exhibits antibacterial, antifungal, antitumor/antileukemic, antiviral, and immunomodulation activities (Cunningham *et al.*, 1950; Seldin *et al.*, 1997; Zhou *et al.*, 2002). To our knowledge there are no reports about the identification of fibrinolytic enzymes in this mushroom. Therefore, we have attempted to find fibrinolytic enzymes in *C. militaris* as well as other medicinal mushrooms. Recently we purified and characterized a fibrinolytic enzyme from the cultured mycelia of *Armillaria mellea* (Lee *et al.*, 2005). In this study we describe the purification and characterization of a fibrinolytic enzyme from the fruiting body of *C. militaris*.

Materials and Methods

Materials

To perform protein purification and characterization *C. militaris* was purchased from the American Type Culture Collection (ATCC), USA. Bovine fibrinogen, bovine thrombin (1,000 units), plasmin (10 units), azocasein, phenylmethyl sulfonylfluoride (PMSF), *N-α-tosyl-l-lysine chloromethyl ketone (TLCK), N*-α-tosyl-l-phenylalanine chloromethyl ketone (TPCK), 4-amidinophenylmethane sulfonylfluoride (APMSF), and polyvinylidine difluoride (PVDF) were purchased from the Sigma-aldrich Co. (USA). DEAE sephadex A-50, Sephadex G-75, and HiLoad 16/60 Superdex 75 were purchased from Pharmacia Biotech (Sweden). ProSieve color protein marker and ProSieve protein marker were purchased from Cambrex Co. (USA). Chromogenic substrates were purchased from Chromogenix Co. (Sweden). Additional chemicals were purchased from Sigma-aldrich Co. (USA). Other reagents were special grade and purchased commercially.

Cultivation of C. militaris

To cultivate a stock culture of *C. militaris,* PDA slants were inoculated with mycelia and incubated at 25°C for 7 days. This culture was then used for the seed culture inoculation. The mycelia were transferred to the seed culture medium by punching out approximately 5 mm^2 of the slants with a sterilized cutter. This seed was inoculated into 500 ml flasks containing 200 ml of synthetic medium (40 g/L glucose, 10 g/L yeast extract, 0.5 g/L KH_2PO_4, 0.5 g/L $K_2HPO_4 \cdot 3H_2O$, and 0.5 g/L $MgSO_4 \cdot 7H_2O$) and incubated at 25°C on a rotary shaker (110 rpm) for 5 days. To cultivate *C. militaris* fruiting bodies, 100 g of silk worm larva were packed into culture bottles and sealed using polypropylene. The bottles were sterilized at 121°C for 90 min, inoculated with the seed of *C. militaris* mycelia, and incubated for 20 days at 25°C. Once the hyphae of *C. militaris* reached the bottom of the culture bottles they were moved to a cold room at 16°C with 95% relative humidity, and placed under an incandescent light of 1,000 Lux to induce primordial formation. The incubator containing *C. militaris* was ventilated four times a day to provide fresh air.

Purification of the fibrinolytic enzyme

Unless otherwise stated all procedures that follow were carried out at 4°C. Once cultivated, the *C. militaris* fruiting bodies were removed and immediately stored in a −70°C freezer. The frozen mushrooms were thawed and homogenized with an equal volume of water in a Kenwood blender for 2 min at maximum speed. The homogenate was centrifuged at 600 ×*g* and 4°C for 30 min. The crude extract was then

placed on ice. An equal volume of pre-chilled ethanol was added to the extract drop-wise with constant stirring, after which the solution continued stirring for an hour. The precipitated protein was removed by centrifugation at 600 ×*g* for 30 min at 0°C, and the clarified ethanol-soluble fraction was returned to the ice. The ethanol concentration of the fraction was increased drop-wise to 70% with constant mixing. Stirring was continued for 1 h and the protein was recovered by centrifugation at 600 ×*g* for 30 min at 4°C. Following the removal of the supernatant the pellets were dried and the protein was re-suspended in optimal buffer for ion exchange chromatography. The insoluble material was then removed by centrifugation at 10,000 ×*g* for 10 min at 4°C. The DEAE sephadex A-50 was swollen with 10 mM Tris-HCl buffer (pH 7.4), and then boiled for 2 h at 90°C and degassed under vacuum conditions to complete the swelling. The active fraction was applied to the prepared DEAE sephadex A-50 column (5 × 10 cm) that was pre-equilibrated with the 10 mM Tris-HCl buffer (pH 7.4), and eluted with a linear gradient of 0 M to 1.0 M NaCl (pH 7.4) at a flow rate of 0.5 mL/min at 4°C. The active fraction was pooled and concentrated by freeze-drying and desalting. The desalted active faction was then dissolved with 10 mM Tris-HCl (pH 7.4) that contained 0.15 M NaCl for gel filtration chromatography. Gel filtration chromatography was performed to further purify the sample using a Sephadex G-75 column (1.5 × 100 cm) with 10 mM Tris-HCl buffer (pH 7.4) containing 0.15 M NaCl at a flow rate of 0.1 mL/min. The active fraction was pooled and concentrated by freeze-drying and desalting. The desalted active fraction was dissolved in 0.05 M phosphate buffer containing 0.15 M NaCl (pH 7.4). FPLC was then performed for further fractioning via a HiLoad 16/60 Superdex 75 column equilibrated with 0.05 M phosphate buffer containing 0.15 M NaCl (pH 7.4) at a flow rate of 1.0 ml/min. The active fractions were pooled and concentrated by Centricon 30,000 (Amicon Co. USA), and analyzed for purity by SDS-PAGE.

Molecular weight determination

The molecular weight of the purified enzyme was determined by SDS-PAGE, fibrin-zymography, and FPLC. SDS-PAGE was carried out according to the methods described by Laemmli *et al.* (1970), using 10.5% polyacrylamide gel stained with Coomassie Blue R-250. Fibrin-zymography was carried out according to the methods of Kim *et al.* (1998). Resolving gel solution (12%) containing 0.12% (w/v) fibrinogen was prepared in a total 10 ml volume and centrifuged to remove any insoluble impurities that were introduced when the SDS stock solution was mixed. Thrombin (1 unit/ml) solution and *N, N, N', N''-tetramethylethylenediamine (TEMED) were added to the gel solution in final* concentrations of 0.1 μunit/ml and 0.028% (v/v), respectively. The purified enzymes were electrophoresed on fibrin gel, washed in 2.5% Triton X-100 solution, and incubated in a bath containing reaction buffer (prepared with 10 mM Tris–HCl (pH 7.4) containing 0.15 M NaCl, and 0.02% NaN_3) at 37°C for 12-15 h. FPLC was performed using a HiLoad 16/60 Superdex 75 column at a flow rate of 1.0 ml/min. A gel filtration protein comprised of glyceraldehydes-3-phosphate dehydrogenase (35 kDa), carbonic anhydrase (29 kDa), trypsinogen-PMSF (24 kDa), and trypsin-inhibitor (20.1 kDa) was used as a marker.

Determination of the N-terminal sequence

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

The Fibrinolytic and fibrinogenolytic assays

Fibrinolytic activity was determined using the method described by Astrup and Mullertz (1952), with minor modifications. The fibrin agarose plate was made of a 1 mm thickness and contained 1.2% agarose, 0.4% fibrinogen, and 20 units/ml of thrombin. The clot was allowed to stand for 1 h at room temperature. Then 10 μl of the sample solution was carefully placed onto the plate. The plate was incubated for 5 h at 37°C and the diameter of the lytic circle was measured. In the fibrin plate method, a clear transparent region is observed where fibrin is hydrolyzed and its diameter is directly proportional to the potency of the fibrinolytic activity. Fibrin degradation analysis was performed by a method that was slightly modified from a method by Datta *et al.* (1995). In brief, 10 μg of human fibrinogen solution (prepared with 10 mM Tris–HCl pH 7.4 containing 0.15 M NaCl) was added to the human thrombin (0.1 NIH unit), and allowed to stand for 1 h at room temperature. Formed clots were mixed with purified enzyme and incubated at 37°C for various time intervals. Plasmin was used as a positive control. The resulting enzymes were analyzed by SDS-PAGE on 12% gel. The fibrinogenolytic activity was measured as follows: 80 μl of 1% fibrinogen (prepared with 20 mM Tris–HCl pH 7.5 containing 0.15 M NaCl) was incubated with 10 μg of the purified enzyme at 37°C; at various intervals a portion of the reaction solution was withdrawn and analyzed by SDS-PAGE according to the method of Laemmli *et al.* (1970). Plasmin was used as a positive control.

The effects of temperature and pH on enzyme activity
The optimal temperature for enzyme activity was determined by measuring residual activity after the incubation of 10 μl of the purified enzyme in 90 μl of 20 mM Tris-HCl (pH 7.5) at different temperatures (20-80°C) for 1 h. The optimal pH for the fibrinolytic activity of the purified enzyme was determined within a pH range of 2-10. A 10 μl amount of the enzyme solution was added to the following buffers: 90 μl of 0.5 M glycine-HCl (pH 2.0-3.0), 0.5 M acetate (pH 4.0-5.0), 0.5 M Tris-HCl (pH 6.0-8.0), and 0.5 M glycine-NaOH (pH 9.0-10.0). After 1 h of incubation at room temperature the remaining protease activity of each enzyme solution was measured with 0.1% azocasein.

The effects of metal ions and protease inhibitors on the enzyme activity
The effects of metal ions on enzyme activity were investigated using $MgCl_2$, $ZnCl_2$, $CoCl_2$, $FeCl_2$, $CaCl_2$, and $CuSO_4$. The purified enzymes were pre-incubated in both the absence and the presence of bivalent cations including Mg^{2+}, Ca^{2+}, Co^{2+}, Cu^{2+}, Zn^{2+}, and Fe^{2+} with a final concentration of 1.0 mM in 10 mM Tris-HCl (pH 7.4) for 1 h at 37°C. After 1 h of incubation at room temperature the residual protease activity was measured with 0.1% azocasein. The effects of the protease inhibitors were also assessed using 5.0 mM EDTA, 2.0 mM PMSF, 0.5 mM TLCK, 0.5 mM TPCK, 0.5 mM APMSF, 0.05 mM aprotinin, and 0.5 mM pepstain A. The enzyme was pre-incubated with these protease inhibitors for 1 h at 37°C. After incubation the effects were assessed with 0.1% azocasein.

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

Results

Purification of the fibrinolytic enzyme
The fibrinolytic enzyme was purified using a combination of chromatographic steps listed in Table 1. The crude extract underwent anion-exchange chromatography on a DEAE Sephadex A-50 column (5 × 10 cm) and the active fractions (fraction No. 120-129) were eluted with a 0.3 M NaCl concentration (Fig. 1A). The active fractions were collected and desalted and then dissolved in 10 mM Tris-HCl (pH 7.4) containing 0.15 M NaCl for further separation via gel filtration chromatography on a Sephadex G-75 column (1.5 × 120 cm). The major fractions (fraction No. 21-27) with fibrinolytic activity were collected (Fig. 1B) and loaded onto a HiLoad 16/60 Superdex 75 column using ACTA FPLC, which yielded one major peak (fraction No. 10-17) showing strong fibrinolytic activity (Fig. 1C). From a 100 g sample of *C. militaris,* 0.96 mg of the enzyme was purified 191.8-fold with a yield of 12.9% (Table 1). SDS-PAGE (Fig. 1D) and fibrin-zymography were employed to verify the purity of the isolated enzyme (Fig. 2B). The molecular mass of the fibrinolytic enzyme was estimated as 52 kDa using ACTA FPLC and a HiLoad 16/60 Superdex 75 size exclusion column (Fig. 2A). This value is similar to the value that was estimated by SDS-PAGE (Fig. 1D) and fibrin-zymography (Fig. 2B).

Table 1. The purified fibrinolytic enzyme activity yields from *C. militaris*

Purification Step	Volume (ml)	Protein (mg)	Proteolytic activity (unit)	Specific activity (unit/mg)	Recovery (%)	Fold
Homogenate	250	ND	ND	-	-	-
Crude extracts	150	1,552	5,122	3.3	(100)	(1)
DEAE Sephadex A-50	30	204	1,848	9.1	3.1	2.8
Sephadex G-75	5	9.2	1,071	116.4	20.9	35.2
HiLoad 16/60 Superdex 75 pg	1.5	0.96	659.5	633	12.9	191.8

Note: ND = not determined. Protease activity was measured by using the azocasein assay, as described under Materials and Methods.

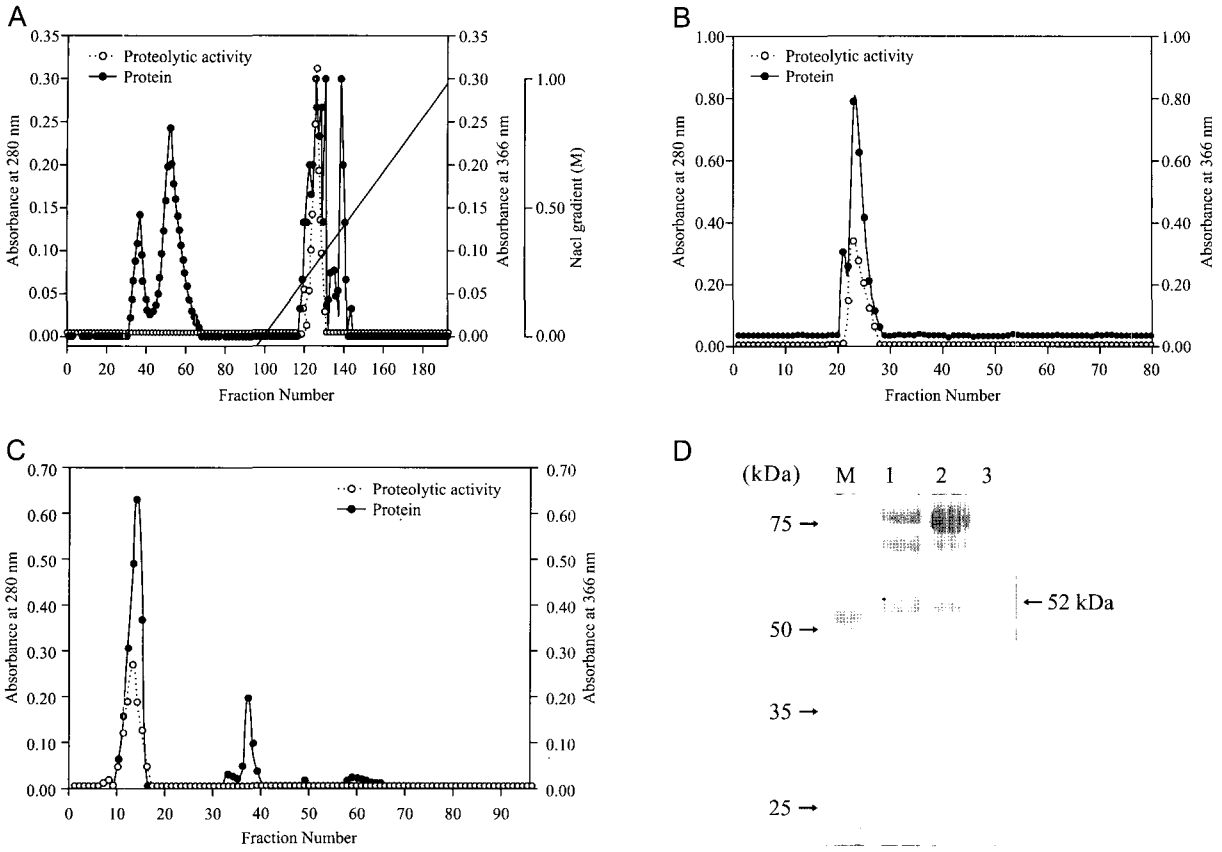

Fig. 1. Purification of the fibrinolytic enzyme from *C. militaris*. (A) Ion-exchange chromatography on a DEAE sephadex A-50 column, (B) gel filtration on a Sephadex G-75 column, (C) The HiLoad 16/60 Superdex 75 column. The elution profiles were monitored by spectrophotometry at 280 nm. Fibrinolytic activity was measured by the azocasein assay at 366 nm. (D) SDS-PAGE was carried out in 10.5% polyacrylamide gel. The protein in the gel was stained with Coomassie brilliant blue R-250. *M*: a protein standard marker; *lane 1*: crude extracts of *C. militaris*; *lane 2*: pooled fractions of the DEAE Sephadex A-50 anion exchange chromatography; *lane 3*: pooled fractions of the HiLoad 16/60 superdex 75 FPLC.

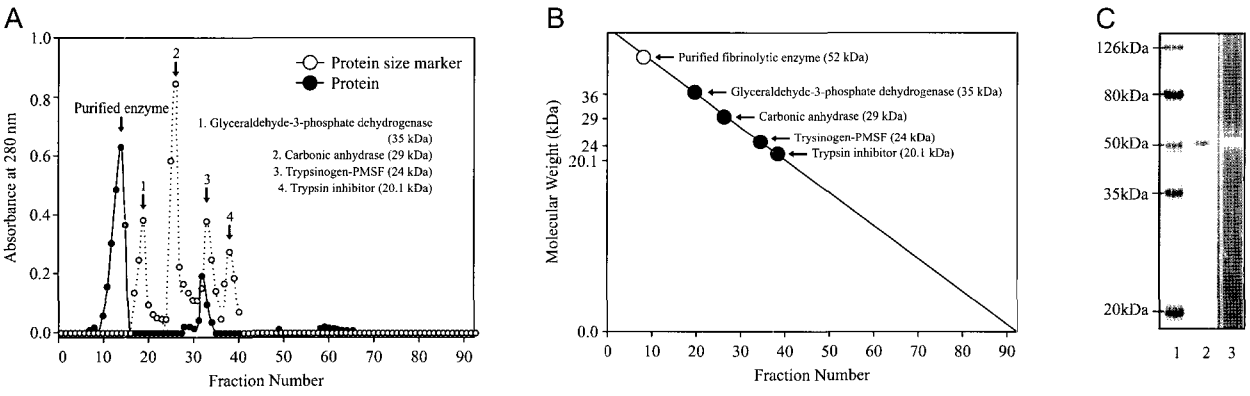

Fig. 2. Molecular weight determination of the fibrinolytic enzyme using size-exclusion on a HiLoad 16/60 superdex 75 column (A), Semi-logarithmic plot (B), and SDS-PAGE and fibrin-zymography (C). (A) The standard marker was eluted through a HiLoad 16/60 superdex 75 column equilibrated with 0.05 mM phosphate buffer containing 0.15 M NaCl, pH 7.4, at a flow rate of 1 ml/min. The elution profiles were monitored by spectrophotometry at 280 nm. (B) The semi-logarithmic plot for size-exclusion. (C) SDS-PAGE and fibrin-zymography. Lane 1: protein size marker; lane 2: the purified fibrinolytic enzyme on the SDS-PAGE; lane 3: the purified fibrinolytic enzyme on the fibrin-zymography.

Purified enzyme	A	L	T	T	Q	S	N	V	T	H	G	L	A	T	I	S	L	R	Q
CAC95048 : Subtilisin PR1J	E	L	T	T	Q	K	N	S	T	H	G	L	A	T	V	S	H	R	E
CAC95046 : Subtilisin PR1B	G	F	V	E	Q	K	N	A	P	W	N	L	A	R	I	S	H	R	Q
CAC63912 : Subtilisin PR1G	G	V	T	T	Q	Q	Q	A	P	W	G	L	A	R	L	S	H	R	R
CAC95049 : Subtilisin PR1A	G	I	T	E	Q	S	G	V	P	W	G	L	G	R	I	S	H	R	Q
CAC07219 : Subtilisin PR1K	A	-	-	T	Q	Q	N	A	D	W	G	L	A	R	L	S	S	Q	K
CAC95043 : Subtilisin PR1I	A	F	A	E	Q	S	G	A	P	W	G	L	S	R	I	S	H	R	R

Fig. 3. Alignment of the N-terminal sequence of the purified fibreinolytic enzyme from *C. militaris* with the subtilicin-like serine protease PR1 family purified from *Metarhizium anisopliae var. anisopliase.*

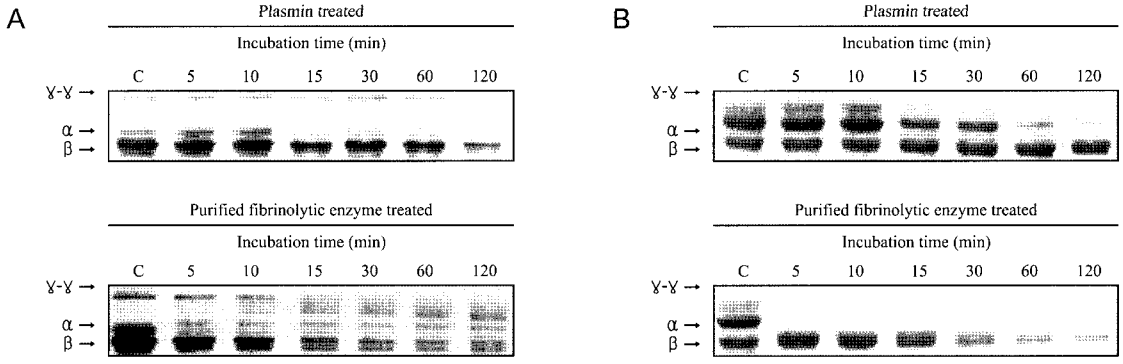

Fig. 4. Fibrinolysis (A) and fibrinogenolysis (B) patterns exhibited by the purified fibrinolytic enzyme over time. Fibrin and fibrinogen were incubated with the purified fibrinolytic enzyme for the times indicated. Plasmin was used as the positive control.

The N-terminal amino acid sequence of the fibrinolytic enzyme

After SDS-PAGE and electroblotting the N-terminal amino acid sequence of the purified fibrinolytic enzyme was analyzed via the automated Edman method. The N-terminal sequence of the first 19 residues was ALTTQSNVTHGLATISLRQ (Fig. 3). This sequence exhibited a high degree of homology to the N-terminal sequences of the subtilisin-like serine protease PR1 family, and specifically to that of PR1J from *Metarhizium anisopliae var. anisopliase* (Gene bank, CAC95048). The sequence alignment showed that the purified enzyme shared the highest (68%) sequence identity with subtilisin PR1J (Gene bank, CAC95048) followed by PR1A (Gene bank, CAC95049). Similarly, the purified enzyme had a 42% sequence homology to PR1B, PR1G, PR1K and PR1I (Gene bank; CAC95046, CAB63912, CAC07219, and CAC95043) from *Metarhizium anisopliae var. anisopliase.*

Analysis of fibrinolysis and fibrinogenolysis

The hydrolysis of fibrin was analyzed by SDS-PAGE. Fig. 4A shows that the purified enzyme rapidly hydrolyzed the fibrin α-chain followed by the γ-γ chains; the β-chain was also hydrolyzed, but more slowly. This hydrolysis pattern is not identical to that of plasmin, which rapidly hydrolyzes the α-chain and more slowly digests the β and γ-γ chains. The purified enzyme also exhibited fibrinogenolytic activity by rapidly hydrolyzing the fibrinogen Aα, Bβ, and γ chains (Fig. 4B).

The effects of pH and temperature on fibrinolytic activity

The effect of pH on the activity of the purified enzyme was determined using buffers at various pH values. Our results indicate that the enzyme was active over a wide pH range (2.0-10.0), but exhibited maximum activity at pH 7.4. We found the enzyme was highly stable in conditions with a pH range of 7.0-8.0 at 37°C for 1 h, and more than 80% of its activity was sustained to a pH of 10.0 (Fig. 5A). The influence of temperature on the enzyme's fibrinolytic activity showed that it was active between 20 and 40°C with an optimum activity at 37°C. However, when it was exposed for 1 h to a temperature greater than 40°C, the fibrinolytic activity decreased dramatically (Fig. 5B).

The effects of inhibitors and metal ions on the fibrinolytic activity

The effect of various inhibitors on the fibrinolytic activity is summarized in Table 2. The purified fi-

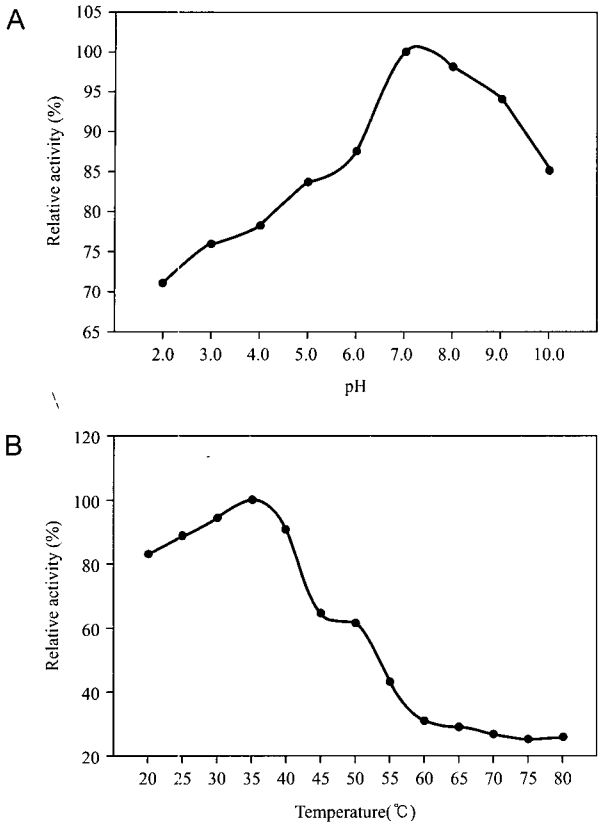

Fig. 5. The effects of pH (A) and temperature (B) on the activity of the fibrinolytic enzyme from *C. militaris*. (A) Enzyme activity was assayed in the pH range of 2-10; 0.5 M glycine-HCl (pH 2.0-3.0), 0.5 M acetate (pH 4.0-5.0), 0.5 M tris-HCl (pH 6.0-8.0), and 0.5 M glycine-NaOH (pH 9.0-10.0) buffers were used with 0.1% azocasein. Enzyme activity was measured by incubation at various pH values and 37 °C. (B) The effects of temperature on the activity of the fibrinolytic enzyme. The purified enzyme was incubated at temperatures from 20 to 80 °C. The fibrinolytic activity was measured by an azocasein assay at 366 nm.

Table 2. The effects of protease inhibitors on the activity of the fibrinolytic enzyme from *C. militaris*. The enzyme was pre-incubated with various protease inhibitors for 1h at 37°C. After incubation the azocasein assay and fibrin plate method (data not shown) were performed. The results are expressed as the relative percentage (%) of relative activity. All experiments were performed in triplicate.

Protease inhibitor	Concentration (mM)	Relative activity (%)
Control	-	100.0 ± 3.5
PMSF	2.0	53.5 ± 2.1
APMSF	0.5	51.5 ± 3.2
TLCK	0.5	73.0 ± 2.5
TPCK	0.5	77.1 ± 2.7
EDTA	5.0	98.2 ± 3.1
Aprotonin	0.05	83.5 ± 2.5
Pepstain A	0.5	87.3 ± 3.7

brinolytic enzyme was inhibited by 2.0 mM PMSF and 0.5 mM APMSF, both of which are well-known serine protease inhibitors. The trypsin selective reagent TLCK and the chymotrypsin alkylating agent TPCK did not inhibit enzyme activity. As shown in Table 3, the effects of various metal ions on enzyme activity were assessed via the residual enzyme activity assay after incubation of the enzyme with 1.0 mM of metal ions for 1 h at 37°C. The enzyme's activity was found to be slightly enhanced by Ca^{2+} and Mg^{2+}, but inhibited by the Co^{2+}, Cu^{2+}, Fe^{2+}, and Zn^{2+} ions.

The amidolytic activity of the fibrinolytic enzyme

The amidolytic activity of the purified fibrinolytic enzyme was assessed with several chromogenic substrates (Fig. 6). The enzyme exhibited the highest degree of specificity for the chymotrypsin substrate S-2586 (MeO-Suc-Arg-Pro-Tyr-pNA · HCl) and was therefore considered to be a chymotrypsin-like serine protease.

Discussion

In this report we have described the purification and characterization of a subtilisin like protease from the medicinal mushroom *C. militaris*. We purified 0.96 mg of the fibrinolytic enzyme from 100 g of *C. militaris*. The purification was 191-fold for a total yield of 12.9%. The molecular weight of the purified enzyme was calculated as 52 kDa by gel filtration, SDS-PAGE, and fibrin-zymography. This was dissimilar to the molecular weights determined for the metalloendopeptidases from *G. frondosa* (20 kDa) (Datta *et al.*, 1995; Nonaka *et al.*, 1995), *A. mellea* (32 kDa) (Kim *et al.*, 1998; Lee *et al.*, 2005) and *P. ostreatus* (19 kDa) (Dohmae *et al.*, 1995; Shin and Choi, 1999; Joh *et al.*, 2004). The N-terminal sequence of the first 19 amino acid residues was ALTTQSNV THGLATISLRQ, which is similar to that of the subtilisin-like serine protease PR1J from *Metarhizium anisopliae var. anisopliase* (Gene bank, CAC95048) (Bagga *et al.*, 2004). Results for the fibrinolysis pattern showed that the enzyme rapidly hydrolyzed the fibrin α-chain followed by the γ-γ chains. It also hydrolyzed the β -chain, but more slowly. We also found that the substrate specificity of the purified enzyme was different from that of plasmin and other proteases that were purified from natural source such as the mushroom *Codium* sp. and snake venom (Matsubara *et al.*, 1998; Siigurkey *et al.*, 1998; Matsubara *et al.*, 1999; Matsubara *et al.*, 2000; Koh *et al.*, 2001; Lee *et al.*, 2005; Bello *et al.*, 2006). The fibrinogenolysis pattern revealed that the enzyme rapidly hydrolyzed the fibrinogen Aα, Bβ, and γ chains. This pattern is dissimilar to that of α-fibibrinogease from snake

Table 3. The effect of metal ions on the activity of the fibrinolytic enzyme from *C. millitaris*. The enzyme was pre-incubated with various metal ions at a concentration of 1 mM for 1 h at 37°C. After incubation the azocasein assay and fibrin plate method (data not shown) were performed. The results are expressed as the relative percentage (%) of relative activity. All experiment was performed in triplicate.

Metalions	Relative activity (%)
Control	100.0 ± 3.5
Cu^{2+}	53.5 ± 2.1
Co^{2+}	51.5 ± 3.2
Ca^{2+}	73.0 ± 2.5
Zn^{2+}	77.1 ± 2.7
Fe^{2+}	98.2 ± 3.1
Mg^{2+}	83.5 ± 2.5

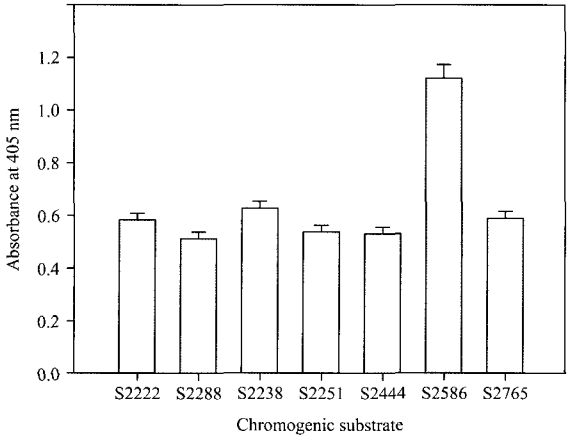

Fig. 6. Amidolytic activity on several chromogenic substrates. Amidolytic activities were measured spectrophotometrically using chromogenic protease substrates.

venom, which preferentially hydrolyzed the Aα chain of fibrinogen rather than the Bβ and γ chains (Pinto *et al.*, 2004; Wei *et al.*, 2005). The purified fibrinolytic enzyme from *C. militaris* is a direct-acting fibrinolytic and fibrinogenolytic agent as it acts via direct cleavage of fibrin and fibrinogen and not by plasminogen activators such as SK, UK, and tPA (Fig. 4). Therefore, secondary effects such as platelet activation related to plasmin formation can be avoided. This is a specific advantage of the *C. militaris* enzyme over clinically used plasminogen activators. The results of our study indicate that this enzyme could be employed for thrombolytic therapy, but also may be used to prevent the formation of venous blood clots.

The optimum pH of the enzyme was 7.4, which is comparable to the pH of FP I and FP II from *Pleurotus sajor-caju* (Shin and Choi, 1999), and MEF from the egg cases of *Tenodera sinensis* (Hahn *et al.*, 1999; Hahn *et al.*, 2001). The optimum temperature for the enzyme was 37°C, but when it was exposed to temperatures over 40°C the fibrinolytic activity degenerated abruptly. Enzyme activity was inhibited by Cu^{2+} and Co^{2+}, but enhanced by the addition of Ca^{2+} and Mg^{2+} ions. The data on the substrate specificity indicate that the purified enzyme is a chymotrypsin-like protease. Also, it more efficiently hydrolyzed the peptide substrate containing four amino acid residues than the substrates containing two or three amino acid residues (Fig. 6). The effects of the inhibitors (Table 2) indicated that the enzyme was a serine protease, and was the least sensitive to TPCK, a specific short substrate-like chymotrypsin inhibitor. This agrees with the enzyme activity it demonstrated with the short amino acid residue substrates. Similar inhibitory effects have been observed in chymotrypsin-like proteinases from insects. For example, chymotrypsin-like proteinases from Coleoptera: *Tenebrio molitor* (Elpidina *et al.*, 2005); Lepidoptera: *Heliothis virescens* (Johnston *et al.*, 1995), *Spodoptera littoralis* (Lee and Anstee, 1995), and *Lacanobia oleracea* (Gatehouse *et al.*, 1999); Orthoptera: *Locusta migratoria* (Lam *et al.*, 1999), *H. zea,* and *A. ipsilon* (Mazumdar-Leighton and Broadway, 2001); and a cockroach *Periplaneta americana* (Baumann, 1990) were insensitive or less sensitive to TPCK.

In conclusion, the fibrinolytic enzyme we obtained from the medicinal mushroom *C. militaris* exhibited profound fibrinolytic activity, and has broad substrate specificity for synthetic peptides. It may be useful for thrombolytic therapy similar to other potent fibrinolytic enzymes such as nattokinase and earthworm enzyme. This enzyme could provide an adjunct to the costly fibrinolytic enzymes that are currently used for managing heart disease since large quantities can be conveniently and efficiently produced. Therefore, *C. militaris* may become a new source of fibrinolytic enzymes for future applications. Lastly, further studies are necessary to elucidate its molecular biological characteristics as well as its medicinal applications.

Acknowledgement

This study was supported by research funds from Chosun University, 2004

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