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Purification and Characterization of Two Novel Fibrinolytic Proteases from Mushroom, *Fomitella fraxinea*

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Abstract Two fibrinolytic enzymes were purified from the culture supernatant of *Fomitella fraxinea* mycelia by ion-exchange and gel filtration chromatographies, and were designated as *F. fraxenia* proteases 1 and 2 (FFP1 and FFP2). The apparent molecular masses of the enzymes were estimated to be 32 kDa and 42 kDa, respectively, by SDS-PAGE and gel filtration chromatography. Both enzymes had the same optimal temperature (40°C), but different pH optima (10.0 and 5.0 for FFP1 and FFP2, respectively). FFP1 was relatively stable at pH 7.0–9.0 and temperature below 30°C, whereas FFP2 was very stable in the pH range of 4–11 and temperature below 40°C. FFP1 activity was completely inhibited by phenylmethylsulfonyl fluoride (PMSF) and aprotinin, indicating that this enzyme is a serine protease. The activity of FFP2 was enhanced by the addition of Co^{2+} and Zn^{2+} and inhibited by Cu^{2+}, Ni^{2+}, and Hg^{2+}. Furthermore, FFP2 activity was strongly inhibited by EDTA and 1,10-phenanthroline, implying that the enzyme is a metalloprotease. Both enzymes readily hydrolyzed fibrinogen, preferentially digesting the Aα- and Bβ-chains of fibrinogen over γ-chain. FFP1 showed broad substrate specificity for synthetic substrates, but FFP2 did not. K_m and V_{max} values of FFP1 for a synthetic substrate, *N*-succinyl-Ala-Ala-Pro-Phe-pNA, were 0.213 mM and 39.68 units/ml, respectively. The first 15 amino acids of the N-terminal sequences of both enzymes were APXXPXGPWGPQRIS and ARPP(G)VDGQ(R,I)SK(L)ETLPE, respectively.

Key words: *Fomitella fraxinea*, fibrinolytic protease, purification, characterization, serine protease, metalloprotease

Fibrin is the primary protein component of a blood clot, which is formed from fibrinogen by thrombin [31]. 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 [7]. The hydrolysis of fibrin is also 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 [9].

Current fibrinolytic agents available for clinical use are mostly plasminogen activators such as a tissue-type plasminogen activator (t-PA), a urokinase-type plasminogen activator, and the bacterial plasminogen activator streptokinase [27, 33]. However, all these agents have undesirable side effects and are very expensive [22]. Therefore, the searches for other fibrinolytic enzymes from various sources are being continued. Sumi *et al.* [29, 30] isolated a fibrinolytic enzyme, nattokinase (NK), from *natto*, a traditional soybean fermented food in Japan, and reported that oral administration of *natto* enhanced fibrinolysis and the production of tPA. Kim *et al.* [14] and Paik *et al.* [26] purified strong fibrinolytic enzymes produced by *Bacillus* sp. strain CK 11-4 and *Bacillus subtilis* KCK-7 from *Chungkook-Jang*, a traditional Korean soybean-fermented food. This enzyme was characterized as a thermophilic alkaline serine protease. In addition, fibrinolytic enzymes from various *Bacillus* sp. stains isolated from *Doen-Jang*, a traditional Korean soybean-fermented food, have been characterized [5, 19].

It is well known that some edible or nontoxic mushrooms contain biologically active substances, showing medicinal effects such as antitumor, antiviral, immunostimulating, antioxidative, and anticoagulating activities [3, 15, 17]. Furthermore, fibrinolytic proteases have been purified and characterized from mycelia and fruit bodies of several edible

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mushrooms, including *Pleurotus ostreatus*, *P. sajor-caju*, *Grifola frondosa*, *Tricholoma saponaceum*, and *Armillaria mellea* [4, 12, 13, 20, 24, 28].

Fomitella fraxinea, which mainly inhabits Korea and East Asia, is one of the medicinal mushrooms. Only a few studies on anticancer or antioxidant activity from *F. fraxinea* fruit bodies have been reported, even though this mushroom is widely used in folk medicine [25]. In an effort to discover fibrinolytic enzymes and develop a therapeutic agent for the treatment of thrombosis, we previously identified a potent fibrinolytic enzyme from the culture supernatant of *F. fraxinea* [18].

In this paper, we describe the purification and characterization of two fibrinolytic proteases from the culture supernatant of *F. fraxinea*.

MATERIALS AND METHODS

Microorganism and Culture Condition

F. fraxinea mycelium was obtained from Korean Agricultural Culture Collection (KACC), Suwon, Korea, and cultured in media supplemented with 2% galactose, 0.6% yeast extract, 0.1% $NaNO_3$, 0.1% K_2HPO_4, and 0.05% $MgSO_4 \cdot 7H_2O$ at 25°C for 10 days according to Lee *et al.* [18]. This culture supernatant was used as the source of the enzyme to carry out the purification and characterization in this study.

Purification of Fibrinolytic Enzyme

Unless otherwise stated, all procedures were carried out at 4°C. Protein concentration was estimated by the Bradford assay [2], using bovine serum albumin as a standard. After cultivation of *F. fraxinea*, the culture broth was centrifuged at 10,000 ×*g* for 10 min, and the culture supernatant was collected. Ammonium sulfate up to 80% saturation was added to the supernatant to precipitate the enzyme, and the resulting precipitate was dissolved in 50 mM Tris-HCl buffer (pH 7.0). The crude enzyme suspension was dialyzed against the same buffer for 12 h, and then concentrated by ultrafiltration using YM10 membrane (Amicon Corp., U.S.A.). The concentrated suspension was applied to an ion-exchange DEAE-sepharose FF column (2.5×30 cm, Amersham Biosciences, Sweden) previously equilibrated with 50 mM sodium acetate buffer (pH 6.0), and the bound proteins were eluted with a linear gradient of 0 to 0.6 M NaCl in the same buffer at a flow rate of 1 ml/min. The fractions containing fibrinolytic activity were pooled and concentrated by freeze-drying. The fractions were applied to a Superdex 200 HR gel filtration column (1.0×30 cm, Amersham Biosciences) preequilibrated with 50 mM Tris-HCl buffer (pH 7.0) and eluted with the same buffer at a flow rate of 0.25 ml/min by an FPLC (fast protein liquid chromatography, Amersham Biosciences) system. Two major peaks with fibrinolytic activity were obtained, which were named FFP1 and FFP2, respectively. The active fractions were further purified by FPLC with a Mono Q column (0.5×5 cm, Amersham Biosciences) using the same buffer and linear gradient of 0–0.5 N NaCl at a flow rate of 0.5 ml/min. The fractions containing the enzyme activity were pooled, concentrated, and used as the purified enzyme preparations.

Molecular Weight Determination

The molecular weights of the enzymes were determined by SDS-PAGE and gel filtration chromatography on Sephacryl S-200 column. SDS-PAGE was carried out according to the method described by Laemmli [16] using 12% gels. After electrophoresis, the gels were stained with Coomassie brilliant blue R-250. The molecular mass markers used were low molecular weight standards (Sigma).

Fibrinolytic and Fibrinogenolytic Assay

Fibrinolytic activity was measured using the fibrin plate method described by Astrup and Mullertz [1] with a slight modification. The fibrin plates were made by pouring a solution composed of 2.5 ml of 1.0% (w/v) human fibrinogen in 0.1 M phosphate buffer (pH 7.4), 7.4 ml of 1.0 % (w/v) agarose, and 0.1 ml of thrombin (100 NIH unit/ml) into a petri dish. The solution in the petri dish was left for 1 h at room temperature to form a fibrin clot layer. Five μl of sample solution was placed onto the plate. The plate was incubated at 37°C for 8 h, and the enzyme activity was quantified by measuring the lysis area on the plate. An equal volume of plasmin solution (1 NIH unit/ml) was used as a positive control.

Fibrigenolytic activity was measured as follows: 100 μl of 1% (w/v) human fibrinogen in 50 mM sodium phosphate buffer (pH 7.0) was incubated with 10 μg of the purified enzymes at 37°C. At various time intervals, a portion of the reaction mixture was withdrawn and analyzed by 12% SDS-PAGE to examine the cleavage pattern of the fibrinogen chains.

Proteolytic Specificity for Plasma Proteins

The purified enzymes (10 μg/ml) were incubated with each of plasma proteins (2 mg/ml) at 37°C for 1 h in 50 mM sodium phosphate buffer (pH 7.0), and the plasma proteins treated with the enzyme were analyzed by 12% SDS-PAGE. The plasma proteins tested were bovine albumin, human albumin, immunoglobulin G (IgG), thrombin, and hemoglobin.

Protease Assay

Protease activity was determined by measuring the release of acid-soluble material from azocasein (Sigma). Thus, 100 μl of the purified enzymes were mixed with 400 μl of 1% (w/v) azocasein in 50 mM sodium phosphate buffer (pH 7.5), and the reaction mixture was incubated at 37°C for 30 min. Next, 500 μl of ice-cold 10% (w/v) of trichloroacetic acid (TCA) was added to the mixture and

then centrifuged at 10,000 ×*g* for 10 min. The absorbance of the supernatant was measured at 340 nm. One unit (U) of protease activity was defined as the amount of the enzyme to increase an absorbance of 0.001 at 340 nm per minute.

Amidolytic Activity

Amidolytic activity was measured spectrophotometrically using the following chromogenic protease substrates; *N*-succinyl-Ala-Ala-Pro-Phe-pNA, *N*-benzoyl-Phe-Val-Arg-*p*NA, *N*-benzoyl-Ile-Glu-Gly-Arg-*p*NA, *N*-benzoyl-Val-Gly-Arg-*p*NA, *N*-succinyl-Ala-Ala-Ala-*p*NA, *N*-benzoyl-Pro-Phe-Arg-*p*NA, N-(*p*-tosyl)-Gly-Pro-Arg-pNA, and N-(*p*-tosyl)-Gly-Pro-Lys-*p*NA. The purified FFP1 (1 μg/200 μl in 50 mM Tris-HCl, pH 7.4) was mixed with 600 μl of 50 mM each of substrate. After continuous measurement for 3 min at 37°C with a temperature-regulated spectrophotometer, the amount of liberated *p*-nitroanilide was determined by measuring the change of absorbance at 405 nm (ε= 9.65× 10^3 $M^{-1}\cdot cm^{-1}$, Tris-HCl, pH 7.4).

pH and Temperature Dependence of the Fibrinolytic Enzyme Activity

The effect of pH on the fibrinolytic activity of the enzyme was estimated at 37°C in three different buffers; 50 mM sodium acetate buffer (pH 4.0–6.0), Tris-HCl buffer (pH 7.0–9.0), and carbonate buffer (pH 10.0–11.0). *N*-Succinyl-Ala-Ala-Pro-Phe-*p*NA was used as a substrate for FFP1 and azocasein for FFP2. pH stability of the enzymes was measured by incubating the enzymes for 1 h at various pHs and 25°C. The effect of temperature on the enzyme activity was measured on the fibrin plate at temperature range of 20–70°C and pH 7.0. To observe the thermal stability of the enzymes, the enzymes were incubated at various temperatures and pH 7.0 for 1 h.

Effects of Metal Ions and Protease Inhibitors on the Activity

The effect of metal ions on the fibrinolytic activity was investigated using $BaCl_2$, $CaCl_2$, $CoCl_2$, $CuCl_2$, $HgCl_2$, $MgCl_2$, $MnCl_2$, $NiCl_2$, $ZnCl_2$, and $AlCl_3$. The effects of the protease inhibitors were also assessed using *N*-tosyl-L-lysine chloromethyl ketone (TLCK), phenylmethanesulfonyl fluoride (PMSF), *N*-(trans-epoxysuccinyl-L-leucineamino-(4-guanidino)butylamide (E-64), ethylenediaminetetraacetic acid (EDTA), aprotinin, and pepstatin A. The enzymes were preincubated with various inhibitors in 0.1 M McIlvaine buffer (pH 7.0) for 1 h at 40°C. After incubation, the remaining activity of FFP1 and FFP2 was measured by using a synthetic substrate, *N*-succinyl-Ala-Ala-Pro-Phe-*p*NA, and azocasein as substrate, respectively.

Determination of Kinetic Constants

The kinetic constants, K_m and V_{max}, of FFP1 were determined by Lineweave-Burk plot with *N*-succinyl-Ala-Ala-Pro-Phe-*p*NA as a substrate. The reactions were performed in 50 mM Tris-HCl buffer (pH 7.4).

N-Terminal Amino Acid Sequence Analysis

After SDS-PAGE, the purified enzyme on the gel was transferred to a polyvinylidine difluoride (PVDF) membrane by electroblotting, and stained with Coomassie blue. The stained band was excised and used for N-terminal sequencing directly by the automated Edman degradation method using a Procise 491 HT protein sequencer (Applied Biosystem, U.S.A.).

Results and Discussion

Purification of Fibrinolytic Enzymes from *F. fraxinea*

Fibrinolytic enzyme activity was detected in the culture supernatant of *F. fraxinea* mycelia, and the enzymes were purified to electrophoretic homogeneity using chromatographic techniques. The purification steps for the enzymes are summarized in Table 1. After the precipitation and dialysis of the enzyme, chromatographies were performed by FPLC. Two peaks with fibrinolytic activity were obtained on the Superdex 200 HR by gel filtration chromatography (Fig. 1A), and the two fibrinolytic enzymes were designated as *F. fraxinea* proteases 1 and 2 (FFP1 and FFP2). Total

Table 1. Purification table of the fibrinolytic enzymes from *Fomitella fraxinea*.

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification fold
Crude extract	820.94	1,477.70	1.80	100.0	1
Ammonium sulfate (80%)	193.09	868.89	4.50	58.8	2.5
DEAE-Sepharose FF	13.55	356.13	26.28	24.1	14.6
Superdex 200 HR					
FFP1	1.91	135.80	71.10	9.2	39.5
FFP2	4.70	177.52	37.77	12.0	21.0
Mono Q					
FFP1	0.78	67.97	87.14	4.6	48.4
FFP2	1.85	115.26	62.30	7.8	34.6

Note. The protease activity was measured by using azocasein as a substrate, as described under Materials and Methods. An enzyme unit (U) was defined as the amount of enzyme producing acid-soluble material from azocasein to yield an absorbance of 0.001 at 340 nm.

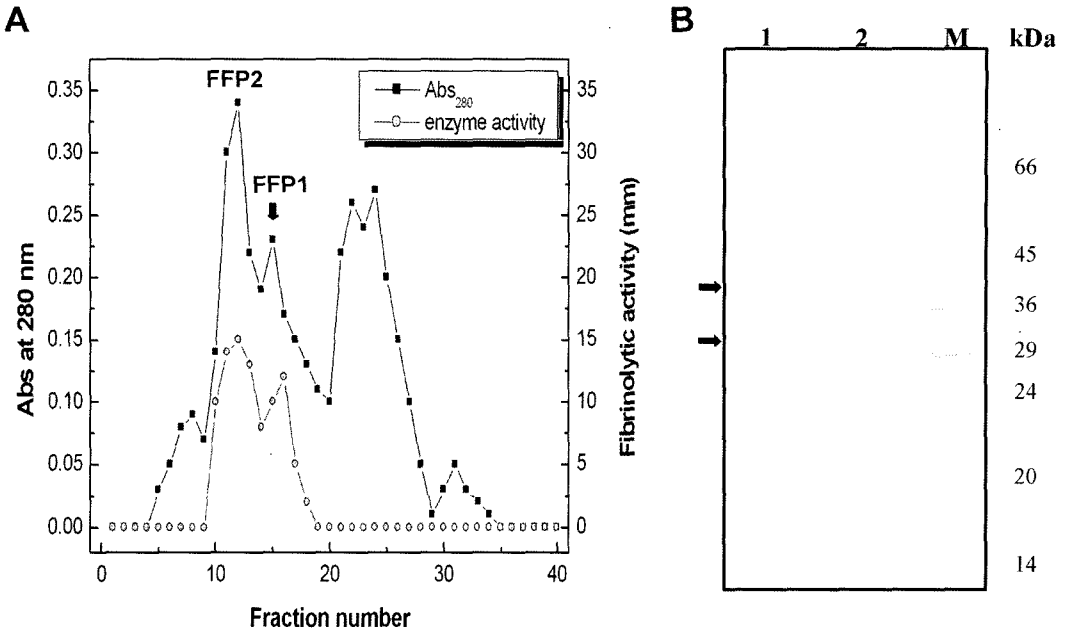

Fig. 1. A. Elution profile of the two fibrinolytic enzymes from *F. fraxinea*, FFP1 and FFP2, by gel filtration chromatography on the Superdex 200 HR column (1.0×30 cm). The enzymes were eluted with 50 mM Tris-HCl buffer (pH 7.4) at a flow rate of 0.25 ml/min by FPLC. **B.** SDS-PAGE of the enzymes.
Lane 1, FFP1; lane 2, FFP2; line M, molecular mass markers.

fibrinolytic enzyme consisted of 29% FFP1 and 71% FFP2 with a recovery of 4.6% for FFP1 and 7.8% for FFP2. These two enzymes were further purified to homogeneity by ion-exchange chromatography on Mono Q. Final purification of the enzymes considerably increased their specific activity. FFP1 from *F. fraxinea* showed a high specific activity of 87.14 U/mg of protein, whereas FFP2 had a relatively low activity of 62.30 U/mg. The purity of the two purified enzymes was verified by the detection of single bands on SDS-PAGE (Fig. 1B).

Molecular Weights of Fibrinolytic Enzyme

The purified enzymes were analyzed by SDS-PAGE and gel filtration to estimate the molecular mass. The apparent molecular weights of FFP1 and FFP2 by SDS-PAGE were 32 kDa and 42 kDa, respectively, as shown in Fig. 1B. In comparison with the value estimated by gel filtration on Sephacryl S-200, using FPLC (data not shown), the two fibrinolytic enzymes from *F. fraxinea* appeared to be monomeric proteins. The molecular weight of FFP1 is similar to that (31 kDa) of fibrinolytic enzyme from *Bacillus subtilis* BK-17 [6], and the molecular weight of FFP2 is the same as the value of the enzyme (42 kDa) from *Bacillus* sp. DJ-2 [5]. On the other hand, the molecular weights of fibrinolytic enzymes from mushrooms *A. mellea* [20] and *G. frondosa* [24] are 21 kDa and 20 kDa, respectively, and the values of the two fibrinolytic enzymes from fruit bodies of *T. saponaceum* are 18 kDa and 18.2 kDa, respectively [13]. Therefore, it is obvious that both fibrinolytic enzymes from *F. fraxinea* have a higher molecular weight than those of other species of mushroom.

Effects of pH and Temperature on Fibrinolytic Activity and Stability

The effects of pH on the activity of purified FFP1 and FFP2 were determined, using buffers with pH values ranging from 4.0–11.0. FFP1 showed the enzyme activity over a wide range of pH (6.0–10.0) being the most active at pH 10.0 (Figs. 2A, 2B). The enzyme was quite stable in the pH range of 7.0–9.0 at 25°C for 1 h. FFP2 was active in the pH range of 5.0–9.0 and exhibited maximum activity at pH 5.0. Furthermore, this enzyme was very stable over a broad pH range of 4.0–11.0. The results showed that the activity and stability of FFP1 rapidly decreased at below pH 6.0, but FFP2 showed high level of activity at acidic pH, exhibiting 85% of its maximum activity at pH 6.0 and 45% of activity at pH 4.0. The optimum temperatures of both enzymes were determined by varying the reaction temperature, and the thermal stabilities of the enzymes were examined by incubating the purified enzymes at different temperatures for 1 h. As shown in Figs. 2C and 2D, FFP1 and FFP2 had an identical optimum temperature of 40°C. However, FFP2 was slightly more thermoresistant than FFP1, exhibiting 79% of the activity at 40°C. At above 40°C, the enzyme activity and stability decreased rapidly, and both enzymes did not show the fibrinolytic activity at above 50°C. Lee *et al.* [20] reported that the optimum pH and temperature of the AMMP from mushroom *A. mellea* were

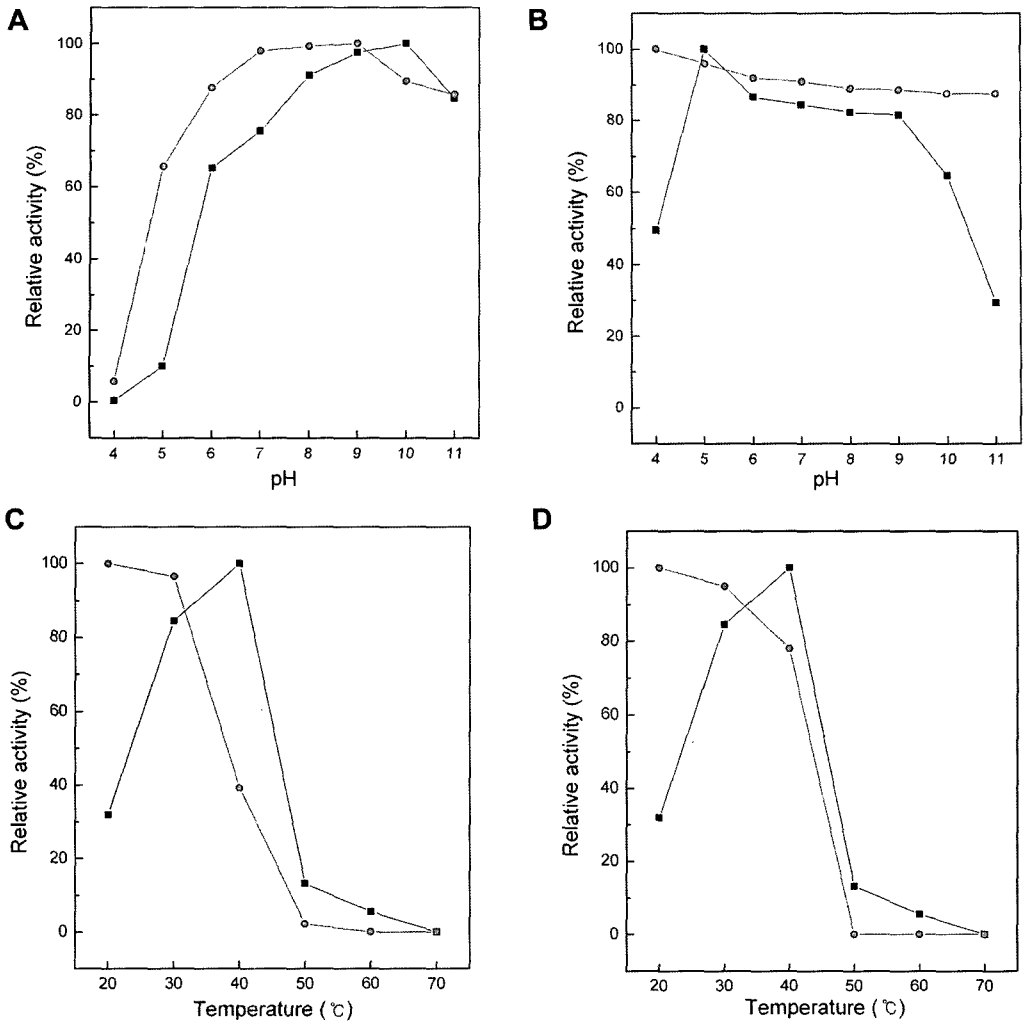

Fig. 2. Effects of pH and temperature on the activities of the purified fibrinolytic enzymes, FFP1 and FFP2.
Effects of pH on the activity and stability of FFP1 (A) and FFP2 (B) from *F. fraxinea*. The enzyme activity (■) was assayed in the pH range 4–11, respectively, in 50 mM sodium acetate buffer for pH 4–6, in 50 mM Tris-HCl buffer for pH 7–9, and in 50 mM carbonate-bicarbonate buffer for pH 10–11 at 40°C. The enzyme stability (●) was measured by incubating it for 1 h at the various pH values and 25°C. Effects of temperature on the activity and thermal stability of FFP1 (C) and FFP2 (D) from *F. fraxinea*. The enzyme activity (■) was assayed at various temperatures of 20–70°C and pH 7 in 50 mM Tris-HCl buffer. The enzyme stability (●) was measured by incubating it for 1 h at the various temperatures and pH 7.0.

pH 6.0 and 33°C, respectively. In the present study, FFP2 from *F. fraxinea* was found to have pH and temperature dependence similar to those of AMMP from *A. mellea*. However, the fibrinolytic enzyme (TSMEP) from *T. saponaceum* [13] had a maximum activity at pH 7.5 and 55°C. Two fibrinolytic enzymes, FPI and FPII, from *P. sajor-caju* [28] exhibited their maximum activity at pH 7.5 and 8.5, respectively, and these enzymes were very stable at 40°C. Therefore, FFP1 was considered to be an alkaline-resistant enzyme and FFP2 to be acid resistant, compared with the fibrinolytic enzymes from other species of mushroom. Moreover, FFP2 was very stable over a broad range of pH values. However, FFP1 and FFP2 from *F. fraxinea* were less stable to heat than fibrinolytic enzymes of other mushrooms.

Effect of Metal Ions and Inhibitors on Fibrinolytic Activity

The effects of metal ions and inhibitors on the fibrinolytic activities of both enzymes are summarized in Table 2. FFP2 was completely inhibited by Cu^{2+}, Ni^{2+}, and Hg^{2+}, but stimulated by Co^{2+} and Zn^{2+}. However, no significant inhibition by metal ions on the FFP1 activity was found. FFP1 was completely inhibited by PMSF and aprotinin, which are known serine protease inhibitors, but TLCK, cysteine protease inhibitor, and pepstatin A, aspartic protease inhibitor, did not affect the enzyme activity. On the other hand, FFP2 was not inhibited by any protease inhibitor, but inhibited by EDTA and 1,10-phenanthroline. In general, EDTA and 1,10-phenanthroline have been known to remove some essential metal ion from the enzyme molecule, resulting in inactivation of an enzyme. The effect of EDTA on the

Table 2. The effects of various metal ions and protease inhibitors on the activity of the fibrinolytic enzymes from *F. fraxinea*.

Metal ions and inhibitors	Concentration (mM)	Relative activity (%)	
		FFP1	FFP2
None	-	100	100
Mg^{2+}	5	84	100
Co^{2+}	5	95	196
Cu^{2+}	5	98	0
Ca^{2+}	5	128	100
Zn^{2+}	5	96	145
Ni^{2+}	5	100	0
Ba^{2+}	5	112	60
Hg^{2+}	5	95	0
Al^{3+}	5	96	75
EDTA[a]	5	97	0
1,10-phenanthroline	1	98	0
PMSF[b]	1	0.3	69
TLCK[c]	1	104	100
E-64[d]	1	70	100
Pepstatin A	0.2	78	100
Aprotinin	0.05	0	100

Enzyme (2 μg) was incubated in 0.1 M McIlvaine buffer (pH 7.0) with different divalent ions and protease inhibitors for 1 h at 40°C. After incubation, the mixture was subjected to the enzyme assay.
[a]Ethylenediaminetetraacetic acid.
[b]Phenylmethylsulfonyl fluoride.
[c]*N*α-tosyl-L-lysine chloromethyl ketone.
[d]*N*-(trans-epoxysuccinyl-L-leucineamino-(4-guanidino)butylamide.

FFP2 activity was further investigated in the presence of various metal ions. FFP2 was inactivated by EDTA and was reactivated by the addition of Zn^{2+} up to 129% (data not shown). From the above, it was strongly suggested that FFP1 from *F. fraxinea* is a serine protease, whereas FFP2 is a metalloprotease requiring Zn^{2+} ion for its catalytic activity. This feature is very similar to other fibrinolytic enzymes. The fibrinolytic enzymes from *Bacillus* sp. CK11-4 [14] and *Bacillus natto* [8] are a serine protease, and the fibrinolytic enzymes from *P. ostreatus* [4], *P. sajor-caju* [28], and *T. saponaeum* [13] are Zn^{2+}-requiring metalloproteases. However, it has been reported that some fibrinolytic enzymes from various sources are lysine-specific proteases or metalloproteases requiring Cu^{2+} or Mg^{2+} [20, 24].

Amidolytic Activity

The amidolytic activity of both enzymes was investigated with several chromogenic substrates (Table 3). FFP1 showed the highest activity for the substrate *N*-succinyl-Ala-Ala-Pro-Phe-*p*NA (for subtilisin or chymotrypsin), and it also showed higher degree of specificity for *N*-benzoyl-Phe-Val-Arg-*p*NA (for trypsin or thrombin). The K_m and V_{max} values of the enzyme for *N*-succinyl-Ala-Ala-Pro-Phe-*p*NA were determined to be 0.213 mM and 39.68 U/ml, respectively (data not shown). However, FFP2 did not show any activity for the synthetic substrates tested. The fibrinolytic enzymes from *Bacillus natto* [8] and *Rhizopus*

Table 3. Amidolytic activity of FFP1 from *F. fraxinea* for several synthetic protease substrates.

Synthetic protease substrates	Substrate hydrolysis (μmol/min/mg)
N-Succinyl-Ala-Ala-Pro-Phe-*p*NA	22.345
N-Benzoyl-Phe-Val-Arg-*p*NA	11.268
N-(*p*-Tosyl)-Gly-Pro-Lys-*p*NA	3.401
N-Benzoyl-Ile-Glu-Gly-Arg-*p*NA	3.131
N-Benzoyl-Val-Gly-Arg-*p*NA	0.975
N-(*p*-Tosyl)-Gly-Pro-Arg-*p*NA	0.321
N-Succinyl-Ala-Ala-Ala-*p*NA	N.D.
N-Benzoyl-Pro-Phe-Arg-pNA	N.D.

N.D., not detected.

*chinesis*12 [21] also exhibited the highest activity for *N*-succinyl-Ala-Ala-Pro-Phe-*p*NA and the K_m values of the enzymes were 0.52 mM and 0.23 mM, respectively. It was found that the K_m value of FFP1 from *F. fraxinea* was lower than that of other enzymes, and FFP1 showed a relatively higher affinity for fibrin hydrolysis. Therefore, it was suggested that the purified enzyme from *F. fraxinea* could be applied as an effective thrombolytic agent.

Fibrinogenolysis and Proteolytic Activity

The hydrolysis of fibrinogen by both enzymes was analyzed by SDS-PAGE (Fig. 3). FFP1 and FFP2 completely hydrolyzed the Aα-chain first in 5 min of incubation, and hydrolyzed Bβ-chain after 20–30 min of incubation. Both enzymes also hydrolyzed the γ-chain, but more slowly. The γ-chain was completely hydrolyzed by FFP1 and FFP2 after 6 h and 12 h of incubation, respectively. AMMP, fibrinolytic enzyme from *A. mellea* [20], preferentially hydrolyzed the Aα-chain, and all of the three subunits chains of fibrinogen were completely hydrolyzed by AMMP after 12 h of incubation. TSMEP1 from *T. saponaceum* [13] also hydrolyzed both Aα-

Fig. 3. Fibrigenolysis by FFP1 (A) and FFP2 (B) from *F. fraxinea*. The purified enzyme (1 μg/ml) was incubated with 1% fibrinogen at 37°C in 50 mM sodium phosphate buffer (pH 7.0). Lanes 1–10 indicate fibrinogen and enzyme after 0, 5, 10, 20, 30, 60, 120, 180, 360 min and 12 h incubation, respectively.

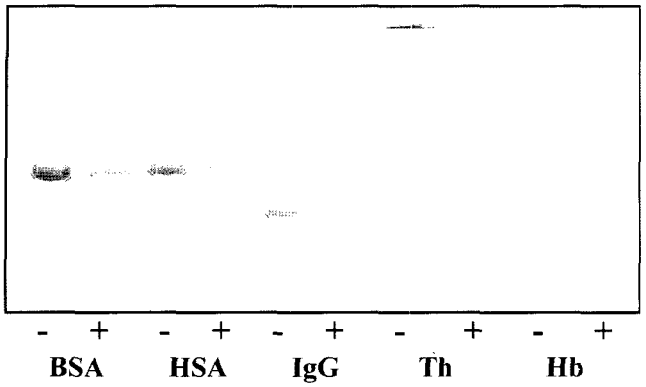

Fig. 4. SDS-PAGE of several plasma proteins in the absence and presence of FFP2 from *F. fraxinea*.
The purified enzyme (10 μg/ml) at 37°C for 1 h in 50 mM sodium phosphate buffer (pH 7.0), bovine serum albumin (BSA), human serum albumin (HSA), immunoglobulin G (IgG), thrombin (Th), and hemoglobin (Hb). (-), not treated enzyme; (+), treated enzyme.

and Bβ- chains with equal efficiency; however, the γ-chain was resistant to hydrolysis by the enzyme, thus indicating that FFP1 and FFP2 from *F. fraxinea* had higher specificity for fibrinogen chains than the enzymes from other species of mushrooms. As shown in Fig. 4, FFP2 did not hydrolyze other blood proteins such human albumin, bovine albumin, IgG, thrombin, or hemoglobin. The proteolytic specificity of FFP2 for blood proteins is quite similar to that of TSMEP1 from *T. saponaceum* [13].

Analysis of N-Terminal Sequences

The N-terminal amino acid sequences of the purified FFP1 and FFP2 were determined by the automated Edman method after SDS-PAGE and electroblotting. The N-terminal sequences of the first 15 residues of FFP1 and FFP2 were APXXPXGPWGPQRIS and ARPP(G)VDGQ(R,I)SK(L) ETLPE, respectively. FFP1 showed 20% (3 of 15 positions) identity with nattokinase [23], amylosacchariticus [32], subtilisin J [10], and subtilisin E [11] from various sources. On the other hand, FFP2 showed no homology with any other fibrinolytic enzymes. In addition, it was observed that N-terminal amino acid sequences of FFP1 and FFP2 from *F. fraxenia* were significantly different from those of fibrinolytic enzymes of other mushrooms such as *A. mella* [20], *T. saponaceum* [13], and *G. frondosa* [23].

It is well known that mushrooms contain medicinal substances as well as nutritious constituents. In this paper, we described the purification and characterization of two fibrinolytic proteases, FFP1 and FFP2, from the culture supernatant of *F. fraxenia*, a medicinal mushroom. Both enzymes exhibit higher hydrolytic activity of fibrin and fibrinogen and significant substrate specificity for some synthetic substrates. Therefore, we suggest that *F. fraxinea* can be a new source to develop therapeutic agents for the treatment of thrombosis as well as other medicines such as antioxidant and antitumor agents.

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