Purification and Characterization of a Fibrinolytic Protease from a Culture Supernatant of *Flammulina velutipes* Mycelia

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In this study we purified a fibrinolytic enzyme from the culture supernatant of *Flammulina velutipes* mycelia by ion exchange and gel filtration chromatographies, it was designated as *F. velutipes* protease (FVP-I). This purification protocol resulted in 18.52-fold purification of the enzyme at a final yield of 0.69%. The molecular mass of the purified enzyme was estimated to be 37 kDa by SDS–PAGE, fibrin-zymography and size exclusion by FPLC. This protease effectively hydrolyzed fibrin, preferentially digesting α-chain over β-and γ-γ chain. Optimal protease activity was found to occur at a pH of 6.0 and a temperature of 20 to 30℃. The protease activity was inhibited by Cu²⁺, Fe²⁺ and Fe³⁺ ions, but was found to be enhanced by Mn²⁺ and Mg²⁺ ions. Furthermore, FVP-I activity was potently inhibited by EDTA and EGTA, and it was found to exhibit a higher specificity for chromogenic substrate S-2586 for chymotrypsin, indicating that the enzyme is a chymotrypsin-like metalloprotease. The first 20 amino acid residues of the N-terminal sequence of FVP-I were LTYRVIPITK-QAVTEGTELL. They had a high degree of homology with hypothetical protein CC1G_11771, GeneBank Accession no. EAU86463.

**Key words:** *Flammulina velutipes*; mycelia; fibrinolysis; fibrinogenolysis; metalloprotease

Intravascular thrombosis due to fibrin aggregation in the arteries is one of the main causes of cardiovascular disease. Fibrin is the primary protein component of blood clots, which are formed from fibrinogen by thrombin.¹ 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.²³ Fibrin clot formation and fibrinolysis are normally well balanced in biological systems. However, when fibrin is not hydrolyzed due to some disorder, thrombosis can occur. Myocardial infarction is the most common form of such thrombosis.

The fibrinolytic agents available today 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.³ Despite widespread use, all these agents have undesired side effects, exhibit low specificity for fibrin, and are also relatively expensive. Therefore, the search for other fibrinolytic enzymes from various sources continues. Over the last decade, potent fibrinolytic enzymes have been discovered from a variety of sources, such as earthworms,⁴ snake venom,⁵ insects,⁶ food-grade microorganisms,⁷ marine creatures,⁸ and fermented food products such as Japanese natto,⁹ Korean chungkook-jang,¹⁰ and Chinese dou-chi.¹¹

In recent years, mushroom have become an attractive source of various physiologically active compounds.¹²–¹⁴ They are commonly used as food and food flavoring substances and also in traditional oriental medicines. Extracts have been reported to exert hematological, antiviral, antitumorigenic, hypotensive, and hepatoprotective effects.¹⁶–¹⁸ These constitute an important source of thrombolytic agents. Some reports have described the fibrinolytic activity of certain edible mushrooms, including *Armillaria mellea*,¹⁹,²⁰ *Grifola frondosa*,²¹ *Pleurotus ostreatus*,²²,²³ and *Cordyceps*.
militaris. The golden needle mushroom, *Flammulina velutipes*, belonging to the family *Tricolomataceae* (Hymenomycetes, Basidiomycota), is quite popular in Korea, Japan, China, and Taiwan. Its extracts have been reported to possess immunomodulatory, anti-tumor, anti-viral, anti-fungal, and cholesterol-lowering properties. Although research has traditionally focused on the therapeutic effects of *F. velutipes*, little information is currently available regarding its fibrinolytic activity. Prior to previous reports, no fibrinolytic enzymes had been isolated from *F. velutipes* cultures. Hence, in this paper we describe the purification and characterization of a fibrinolytic enzyme from the culture supernatant of *F. velutipes*.

**Materials and Methods**

**Materials.** *F. velutipes* strain was provided by the Department of Industrial Crop Production and Processing, Iksan National College, Iksan, Republic of Korea. Fibrinogen, thrombin, and plasmin were purchased from Calbiochem (Darmstadt, Germany). Ethylenediaminetetraacetic acid (EDTA), ethyleneglycol-bis(beta-aminooxylyl ether)-N,N',N'-tetraacetic acid (EGTA), phenylmethylsulfonyl fluoride (PMSF), and 1-chloro-3-tosylamido-7-amo-no-1-s-heptanone (TLCK) were purchased from Sigma (St. Louis, MO, USA). Paranitroanilide (pNA) chromogenic substrates were obtained from Chromogenix (Milano, Italy). CM-cellulose, DEAE sephadex A-50, Sephadex G-75, and HiLoad 16/60 superdex 75 column were purchased from Pharmacia Biotech. (Uppsala, Sweden). All other reagents were of special grade, and are commercially available.

**Mushroom culture and preparation of crude extract.** The mushrooms were cultured in 300 ml Erlenmeyer flasks containing 100 ml of potato dextrose broth (20% potato and 2% dextrose in distilled water) at 25 °C with rotation. The culture broth was separated by filtration from the mycelia. The culture filtrate was freeze-dried and ground to a powder. The powdered samples were homogenized in 10 mM Tris–HCl buffer (pH 7.4) equilibrated with the same buffer. Elution was carried out at a flow rate of 1.5 ml/min. The fractions (1.5 ml of each) were collected and measured for absorbance at 280 nm and protease activity by azocasein assay, as described above. Fractions evidencing high degrees of protease activity were loaded onto a DEAE Sephadex A-50 column (3.5 x 10 cm) equilibrated with the same buffer. Elution was performed at a flow rate of 0.5 ml/min, with a linear gradient of 0–1.5 M HCl, in the same buffer. Fractions exhibiting high levels of protease activity were combined and dialyzed against 10 mM Tris–HCl buffer, pH 6.8. Then the active fractions were concentrated by freeze-drying and applied to a Sephadex G-75 column (1.5 x 130 cm) equilibrated with 10 mM Tris–HCl buffer at pH 7.4, containing 0.15 M NaCl. Elution was performed at a flow rate of 0.1 ml/min, and 1.2 ml fractions were collected. Active fractions were collected and concentrated by freeze-drying. For further purification, FPLC was performed by HiLoad 16/60 Superdex 75 column (Pharmacia Biotech, Uppsala, Sweden) equilibrated with 50 mM phosphate buffer containing 0.15 M NaCl (pH 7.4) at a flow rate of 1.0 ml/min. The active fractions were pooled.

**Determination of the N-terminal amino acid sequence of the protease.** After SDS–PAGE, purified protease on polyacrylamide gel was transferred to a polyvinylidene difluoride membrane (PVDF) by electroblotting, then stained with Ponceau S solution and the stained portion was excised. The N-terminal amino acid sequence of the purified FVP-I was determined using an Applied Bio-
system Precise 491 amino acid sequencer at the Korea Basic Science Center in Seoul, Korea. The sequenced data and sequence alignment were analyzed by BLAST search in the NCBI protein database with default parameters.

Molecular weight determination. The molecular weight of the FVP-I was determined by SDS–PAGE and fibrin-zymography with standard markers (Lonza, Basel, Switzerland). SDS–PAGE was carried out according to the method of Laemmli\(^{28}\) using 12% polyacrylamide gel, the gel stained with Coomassie Brilliant Blue R-250. Fibrin-zymography was carried out according to the method of Kim et al.\(^{29}\) Resolving gel solution (12%) containing 0.12% (w/v) fibrinogen was prepared. Thrombin (1 unit/ml) and TEMED (\(N, N, N, N\)-tetramethyl-ethylenediamine) were added to the gel solution in final concentrations of 0.1 unit/ml and 0.028% (v/v) respectively. Purified FVP-I was electrophorized into fibrin gel and subsequently washed in 2.5% Triton X-100 solution, and incubated in a bath containing such substrates, shown in Table 1. The enzyme activity was summarized in Table 2. The extract was first applied to the method of Laemmli.\(^{28}\)

Fibrinolytic and fibrinogenolytic assays. Fibrin degradation analysis was performed by a slight modification of the method of Datta et al.\(^{30}\) 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 human thrombin (0.1 NIH unit) and allowed to stand for 1 h at room temperature. Formed clots were mixed with purified FVP-I and incubated at 37 °C for various time intervals. Plasmin was used as a positive control. The resulting FVP-I were analyzed by SDS–PAGE on 12% gel. The fibrinogenolytic activity was measured as follows: 80 µg 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 and at various intervals a portion of the reaction solution was withdrawn and analyzed by SDS–PAGE according to the method of Laemmli.\(^{28}\)

Effects of temperature and pH on protease activity. The effects of temperature and pH on the fibrinolytic activity of FVP-I were assessed using the fibrin plate and azocasein assay. The optimal temperature for enzymatic activity was determined by measurement of residual activity after 1 h of incubation of 10 µg of fibrinolytic enzyme with 90 µl of 20 mM Tris–HCl (pH 7.5) at different temperatures (10–80 °C). The optimal pH for the fibrinolytic activity of the enzyme was determined to be within a pH range of 2 to 10. Purified protease (10 µg) was added to 90 µl of 0.5 M Glycine–HCl (pH 2.0 and pH 3.0), 0.5 M Acetate (pH 4.0 and pH 5.0), 0.5 M Tris–HCl (pH 6.0 and pH 8.0), or 0.5 M Glycine–NaOH (pH 9.0 and pH 10.0) buffer, and then incubated for 1 h at 37 °C.

Effect of metal ions and protease inhibitors on protease activity. The effects of metal ions were investigated using MgCl\(_2\), ZnCl\(_2\), CoCl\(_2\), FeCl\(_3\), CaCl\(_2\), LiCl\(_2\), CsCl\(_2\), and CuSO\(_4\). The effects of protease inhibitors were also studied using PMSF (phenylmethyl-sulfonyl fluoride), TLCK (N-alpha-tosyl-L-lysine chloromethyl ketone), TPCK (N-alpha-tosyl-L-phenylalanine chloromethyl ketone), aprotinin, EDTA (ethylenediamine tetraacetic acid), EGTA (Ethyleneglycol-bis(beta-aminoethyl ether)-\(N, N, N, N\)-tetraacetic acid), and pepstatin A. The purified protease was pre-incubated in the absence and presence of bivalent cations, such as Ca\(^{2+}\), Co\(^{2+}\), Cu\(^{2+}\), Fe\(^{3+}\), Fe\(^{2+}\), K\(^+\), Mg\(^{2+}\), Mn\(^{2+}\), Na\(^+\), and Zn\(^{2+}\) at a final concentration of 1 mM in 10 mM Tris–HCl (pH 6.0) for 1 h at 37 °C. After incubation, the residual protease activity was assessed using 1% azocasein.

Amidolytic activity of purified protease. The amidolytic activity of the enzyme was measured spectrophotometrically using various synthetic chromogenic substrates, shown in Table 1. The enzyme activity was evaluated by mixing 1 µg of the purified protease 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 change in the sample at 405 nm following continuous measurements for 5 min at 37 °C.

Results

Purification and N-terminal sequence of the fibrinolytic protease

Fibrinolytic protease activity was detected in a crude extract of the culture broth of \(F.\ velutipes\), and the protease was purified by the fibrin plate method as an assay. The steps in the purification of the protease are summarized in Table 2. The extract was first applied to ion-exchange chromatography on CM-cellulose, and active fractions were obtained (Fig. 1A). These active fractions were further fractionated on a DEAE Sephadex A-50 column. Elution was performed with a linear NaCl gradient, from 0 to 1.5 M, at a flow rate of 1.5 ml/min. The fractions exhibited fibrinolytic activity at a gradient of 0.6 M NaCl (Fig. 1B). These fractions were further purified by gel filtration (Fig. 1C) on a Sephadex G-75 and FPLC on a HiLoad 16/60 superdex 75 column by ÄCTAFPLC (Pharmacia Biotech, Uppsala, Sweden) (Fig. 1D). As can be seen in Table 2, 0.68 mg of enzyme was purified 18.52-fold, with a yield of 0.69%.
SDS–PAGE and fibrin-zymography were employed to verify the purity of the isolated enzyme. The purified protease was designated *Flammulina velutipes* protease I (FVP-I). Its molecular weight was estimated by size-exclusion (Fig. 2A and B), SDS–PAGE, and fibrin zymography to be approximately 37 kDa (Fig. 2C). The purified FVP-I was different from that determined for the 20 kDa of GfMEP from *Glifora frondosa*,21) 19 kDa of PoMEP purified from *Pleurotus ostreatus*,22) 21 kDa of AmMP purified from *Armillaria mellea*,20) and 52 kDa of fibirnolytic enzyme purified from *Cordyceps militaris*.24) As shown in Fig. 2C, the crude extract of *F. velutipes* culture broth contained six proteases that exhibited some degree of fibrinolytic activity. The N-terminal amino acid sequence of the purified FVP-I was analyzed by the automated Edman method in Korea Basic Science Center. As shown in Fig. 3, the first 20 amino acid residues of the N-terminal sequence of FVP-I were LTYRVIPITKQAVTEGTELL, which is very similar to that of the hypothetical protein CC1G11771 (NCBI GeneBank Accession no. EAU86463) purified from *Coprinopsis cinerea* okayama.

Fibrinolytic and fibrinogenolytic activity of FVP-I

The hydrolysis of fibrin by purified enzyme was analyzed by SDS–PAGE. As shown in Fig. 4A, FVP-I rapidly hydrolyzed the Αα- and Ββ-chain more than plasmin treated in fibrinogen, which is fibrin precursor.
Fig. 2. Molecular Weight Determination of FVP-I Using Size-Exclusion on a HiLoad Superdex 75 Column (A), 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 50 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, Semi-logarithmic plot for size-exclusion; C, SDS–PAGE and fibrin-zymography; M, protein size marker; lane 1, purified FVP-I on SDS–PAGE; lane 2, purified FVP-I on fibrin-zymography; lane 3, crude extracts of Flammulina velutipes on fibrin-zymography.

Table 2. Purification Table of the Fibrinolytic Enzyme from Flammulina velutipes

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein (mg)</th>
<th>Total proteolytic activity (unit)</th>
<th>Specific activity (unit/mg)</th>
<th>Yield (%)</th>
<th>Purification fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>99.20</td>
<td>772</td>
<td>7.78</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Cation-exchange chromatography</td>
<td>65.25</td>
<td>670</td>
<td>10.27</td>
<td>65.8</td>
<td>1.32</td>
</tr>
<tr>
<td>Anion-exchange chromatography</td>
<td>4.79</td>
<td>312</td>
<td>65.14</td>
<td>4.8</td>
<td>8.37</td>
</tr>
<tr>
<td>Gel filtration chromatography</td>
<td>0.89</td>
<td>103</td>
<td>115.73</td>
<td>0.9</td>
<td>14.88</td>
</tr>
<tr>
<td>Fast protein liquid chromatography</td>
<td>0.68</td>
<td>98</td>
<td>144.12</td>
<td>0.69</td>
<td>18.52</td>
</tr>
</tbody>
</table>

The protease activity was measured using azocasein as a substrate, as described in "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 0.1 at 366 nm, following 1 h of incubation at 37°C.

Fig. 3. Alignment of the N-Terminal Sequence of Purified FVP-I from Flammulina velutipes with Hypothetical Protein CC1G_11771 (NCBI Genebank Accession no. EAU86463) Purified from Coprinopsis cinerea Okayama 7#130, Which Is Fungalysin Metallopeptidase.

Fig. 4. Fibrinogenolysis (A) and Fibrinolysis (B) Patterns Exhibited by the Purified FVP-I Over Time. Fibrin and fibrinogen were incubated with purified FVP-I for the durations indicated. Plasmin was used as the positive control.
Flammulina velutipes

This hydrolysis pattern of FVP-I was not identical to that of plasmin, which can rapidly hydrolyze the Aα- and Bβ-chain, and more slowly digest γ-γ chains. Also, purified FVP-I had fibrinolytic activity, and the degradation pattern of fibrin by purified FVP-I was analyzed by SDS-PAGE (Fig. 4B). As shown in Fig. 4B, the purified enzyme rapidly hydrolyzed the fibrin α-chain followed by the γ-γ chain and the β-chain was also hydrolyzed, but more slowly. This hydrolysis pattern is similar to that of the plasmin.

Effects of pH and temperature on FVP-I

The effect of pH on the activity of purified FVP-I was determined using buffers at various pH values. The effect of temperature on the fibrinolytic activity revealed that the protease was active between 20°C and 60°C. Optimum activity was found to occur at 30°C. However, when exposed for 1 h to a temperature of over 40°C, the fibrinolytic activity of FVP-I decreased radically (Fig. 5A). These results indicate that FVP-I was active over a wide pH range (3.0 to 10.0), but exhibited maximum activity at pH 6.0. The protease was partially stable in a pH range of 5.0 to 8.0 at 37°C for 1 h, but above pH 8.0, protease stability degenerated abruptly (Fig. 5B).

Effect of inhibitors and metal ions on FVP-I

The effect of metal ions and various protease inhibitors on fibrinolytic activity is summarized in Tables 3 and 4. The effects of various metal ions on protease activities after incubation of the protease with 1 mM metal ions for 1 h at 37°C. After incubation come the azocasein assay. The results are expressed as the relative percentage (%) of relative activity.

This was pre-incubated with various metal ions at a concentration of 1 mM for 1 h at 37°C. After incubation, azocasein assay was performed. The results are expressed as the relative percentage (%) of relative activity.

Table 3. Effect of Metal Ions on the Activity of FVP-I from Flammulina velutipes

<table>
<thead>
<tr>
<th>Metal ions</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100 ± 1</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>107.3 ± 0.5</td>
</tr>
<tr>
<td>Cu²⁺</td>
<td>69.5 ± 1.3</td>
</tr>
<tr>
<td>Cu²⁺</td>
<td>102.2 ± 0.1</td>
</tr>
<tr>
<td>Fe³⁺</td>
<td>36.4 ± 0.3</td>
</tr>
<tr>
<td>Fe³⁺</td>
<td>36.1 ± 0.1</td>
</tr>
<tr>
<td>K⁺</td>
<td>22.0 ± 0.6</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>107.8 ± 1.1</td>
</tr>
<tr>
<td>Mn²⁺</td>
<td>113.2 ± 0.8</td>
</tr>
<tr>
<td>Na⁺</td>
<td>113.5 ± 0.9</td>
</tr>
<tr>
<td>Zn²⁺</td>
<td>108.8 ± 0.6</td>
</tr>
<tr>
<td>Zn²⁺</td>
<td>70.9 ± 0.6</td>
</tr>
</tbody>
</table>

FVP-I was pre-incubated with various protease inhibitors for 1 h at 37°C. After incubation come the azocasein assay. The results are expressed as the relative percentage (%) of relative activity.

Table 4. Effects of Protease Inhibitors on the Activity of FVP-I from Flammulina velutipes

<table>
<thead>
<tr>
<th>Protease inhibitor</th>
<th>Concentration (mM)</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>100 ± 2.2</td>
</tr>
<tr>
<td>PMSF</td>
<td>2.0</td>
<td>91.6 ± 1.9</td>
</tr>
<tr>
<td>TLCK</td>
<td>0.5</td>
<td>92.0 ± 0.8</td>
</tr>
<tr>
<td>TPCK</td>
<td>0.5</td>
<td>88.8 ± 2.0</td>
</tr>
<tr>
<td>Aprotinin</td>
<td>5.0</td>
<td>98.9 ± 1.1</td>
</tr>
<tr>
<td>EDTA</td>
<td>0.05</td>
<td>9.5 ± 1.2</td>
</tr>
<tr>
<td>EGTA</td>
<td>0.05</td>
<td>8.2 ± 1.3</td>
</tr>
<tr>
<td>Pepstatin A</td>
<td>0.5</td>
<td>78.6 ± 1.7</td>
</tr>
</tbody>
</table>

The enzyme was pre-incubated with various metal ions at a concentration of 1 mM for 1 h at 37°C. After incubation, azocasein assays were performed. The results are expressed as the relative percentage (%) of relative activity.

Effects of inhibitors and metal ions on FVP-I

The effect of metal ions and various protease inhibitors on fibrinolytic activity was summarized in Tables 3 and 4. The effects of various metal ions on protease activity were investigated by assayng residual protease activities after incubation of the protease with 1 mM metal ions for 1 h at 37°C. Protease activities were found to be enhanced by the addition of Ca²⁺ and Mg²⁺, but were inhibited by the addition of Co²⁺, Cu²⁺, Fe³⁺, and Zn²⁺. FVP-I was completely inhibited by the addition of EDTA and EGTA, well-known inhibitors of metalloproteases. FVP-I was partially inhibited by PMSF, TLCK, TPCK, aprotinin, and pepstatin A.

Amidolytic activity of FVP-I

The amidolytic activity of purified fibrinolytic enzyme was assessed with several chromogenic substrates.
As shown in Fig. 6, the fibrinolytic enzyme exhibited a higher degree of specificity for substrate S-2586 for chymotrypsin (MeO-Suc-Arg-Pro-Tyr-pNA-HCl). Thus purified FVP-I was identified as a chymotrypsin-like metalloprotease.

**Discussion**

It is widely known that snake venom is the most abundant source of fibrinolytic enzymes. In recent years, however, fibrinolytic enzymes have been discovered in a variety of organisms. Currently, relatively few reports focus on the fibrinolytic enzymes in mushrooms. Mushrooms are now eliciting increasing attention, as they are a nutritious food with health-stimulating properties and medicinal effects. Here we describe the purification and characterization of FVP-I from a *F. velutipes* culture broth. Six fibrinolytic proteases were isolated. Among these, only one was purified successfully. Isolation of this fibrinolytic enzyme was carried out by a combination of various column chromatographic steps (Fig. 1A–D). Protease activity was used as an index of purification. In ion-exchange chromatography, the protease activity was eluted as several peaks. The peaks were further purified by gel filtration chromatography on Sephadex G-75. The major fractions with fibrinolytic activity were collected and applied to a HiLoad 16/60 Superdex 75 column using AëCTA fast FPLC, which yielded one major peak showing strong fibrinolytic activity. The final recovery rate was about 0.69%, and the purification factor increased 18.52-fold. The specific activity of the purified FVP-I was estimated to be 144.12 U/mg, as determined by the ratio of protease activity (U) to total protein (mg). Protease activity was measured using azocasein as a substrate, as described in “Materials and Methods.”

The molecular weight of FVP-I was calculated to be 37 kDa by SDS-PAGE and fibrin-zymography, similar to katsuwokinase (38 kDa) but almost twice the amount of metalloendopeptidases from *G. frondosa* (20 kDa), *P. ostreatus* (19 kDa), *A. mellea* (21 kDa). The optimum temperature for the activity of FVP-I was found to be 30 °C, similar to that of MEF (30 °C) from the egg cases of *Tenodera sinensis*. FVP-I was active between 20 and 60 °C, although the fibrinolytic activity of FVP-I decreased abruptly at temperatures of over 40 °C. The optimum pH for the activity of FVP-I was 6.0, comparable to the optimum pH for the fibrinolytic activities (FPI and II) of *Pleurotus sajor-caju*, MEF from the egg cases of *Tenodera sinensis*, and AMMP from *A. mellea*. Results for the fibrinolysis pattern showed that the purified enzyme rapidly hydrolyzed the fibrin α-chain, followed by the γ-γ chain. The β-chain was also hydrolyzed, but more slowly. We also found that the purified FVP-I was different from other proteases purified from natural sources, such as *Codium sp.*, mushrooms, and snake venom.

The fibrinogenolysis pattern revealed that the enzyme readily digested the Αα- and Ββ-chains, and also digested the γ-chain, although much more slowly. However, all three-subunit chains of fibrinogen were completely hydrolyzed by FVP-I after 4 hr of incubation. Its degradation pattern proved to be similar to that of AmMEP, MEF, and TSMEPI, but quite dissimilar to that of the fibrinolytic metalloenzyme found in *F. velutipes* fruit bodies. In that case, the Ββ- and γ-chains were digested as incubation time progressed, but the Αα-chain remained undigested throughout the process. The purified fibrinolytic enzyme from the culture supernatant of *F. velutipes* mycelia 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). Thus secondary effects such as platelet activation related to plasmin formation can be avoided. This is a specific advantage of the FVP-I enzyme over clinically used plasminogen activators. The results of our study indicate that this enzyme can be employed in thrombolytic therapy, but also may be used to prevent the formation of venous blood clots.

Enzyme activity was inhibited by Cu²⁺, Fe²⁺, Fe³⁺, and Co²⁺, but was augmented by the addition of Ca²⁺ and Mg²⁺ ions. Furthermore, it was strongly inhibited by EDTA and EGTA, and partially inhibited by PMSF, TLCK, TPCK, aprotonin, and pepstatin A, indicating that the enzyme is a metalloprotease. Markland reported that most α-fibrinogenases found in snake venom are metalloproteinases, and thus their activity can be inhibited by EDTA. The N-terminal sequence of the first 20 amino acids of FVP-I was determined to be similar to that of the hypothetical protein CC1G,11771 (NCBI GeneBank Accession no. EAU86463), a metallopeptidase purified from *Coprinopsis cinerea* okayama. It was observed that, the N-terminal amino acid sequences of FVP-I from *F. velutipes* were significantly different.
from those of fibrinolytic enzymes, of other mushrooms such as A. mellea,20 C. militaris,24 T. saponaceum,46 and G. frondosa.21 In conclusion, the fibrinolytic protease FVP-I, obtained from the culture broth of the edible and medicinal mushroom F. velutipes, exhibits a remarkable degree of fibrinolytic activity. Therefore, this mushroom constitutes a new source of fibrinolytic enzymes for future applications.

Acknowledgments

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