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Research Article

Purification of an Intracellular Fibrinolytic Protease from *Ganoderma Lucidum* Vk12 and its Susceptibility to Different Enzyme Inhibitors

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Abstract

Purpose: *To study the effect of different inhibitors on the fibrinolytic activity of the enzyme produced by Ganoderma lucidum.*
Method: *The intracellular fibrinolytic protease produced by Ganoderma lucidum VK12 was isolated from the mycelia grown in MCDBF broth and was purified to homogeneity using ammonium sulfate fractionation, ion exchange chromatography and sephadex G-150 column chromatography techniques. The purity of the enzyme was verified on SDS-PAGE after silver nitrate staining. The inhibitory effect of different metal ions and commercial protease inhibitors on enzyme activity was studied. The inhibitor-treated enzyme was assayed with its substrate and the residual activity of the enzyme recorded.*
Result: *The fibrinolytic enzyme isolated from Ganoderma lucidum was purified to near homogeneity and it appeared as a single protein band on SDS-PAGE. Metal ions such as Ca^{2+} and Mg^{2+} inhibited the activity of the enzyme while Zn^{2+} ions enhanced the activity. . Complete inactivation occurred when the enzyme was incubated with protease inhibitors such as EDTA, 1, 10-phenanthroline, phosphoamidon while the enzyme was insensitive to protease inhibitors such as leupeptin, PMSF, TPCK and APMSF.*
Conclusion: *Copper sulfate completely inhibited the enzyme activity. The sensitivity of this enzyme to EDTA suggests that it might be a metalloprotease.*

Keywords: *Ganoderma lucidum, Fibrinolytic protease, Protease inhibitors, Copper sulfate, EDTA*

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INTRODUCTION

Blood clots are formed from fibrinogen by thrombin (EC 3.4.21.5) and are lysed by plasmin (EC 3.4.21.7), which is activated from plasminogen by tissue plasminogen activator (tPA). Although fibrin clot formation and fibrinolysis are maintained in balance by the biological system, thromboses such as myocardial infarction, occur when clots are not lysed as a result of a disorder in the balance. Intravenous administration of urokinase and streptokinase was widely used for thrombosis therapy but these enzymes have low specificity for fibrin. tPA was developed for the treatment of thrombosis because of its efficiency and strong affinity for fibrin [1]. However, these enzymes are expensive, and patients may suffer from undesirable side effects such as gastrointestinal bleeding and allergic reaction [2]. Therefore, this necessitated the search for thrombolytic agents from other biological sources. Recently, fibrinolytic enzymes have been discovered in fermented food products such as Japanese natto [3], Korean Chang kook-Jang soy sauce [4] and fermented shrimp paste [5].

In the East Asian countries, the species of *Ganoderma* are regarded as the herb of longevity and have been used in folk medicine for hundreds of years. Some strains have been cultivated commercially for the preparation of health tablets [6]. Medicinal benefits of *Ganoderma* spp. have been reviewed by Jong and Birmongham [7]. Choi and Sa [8] have reported the presence of fibrinolytic enzyme from a Chinese isolate of *Ganoderma lucidum*. In the present study, different *Ganoderma lucidum* isolates collected from several locations in southern India were screened for the presence of fibrinolytic protease enzyme. A strain, VK12, isolated from *Tamarindus indica* which showed the highest fibrinolytic protease activity, was purified, and the effect of different protease inhibitors on the activity of the enzyme was been evaluated in this study.

EXPERIMENTAL

Organism and culture conditions

The isolates of *Ganoderma lucidum* were collected from different ecological niches of Southern India and the pure cultures were prepared and maintained in potato dextrose agar medium (PDA). All the isolates were screened for the production of fibrinolytic protease by growing them on the fibrin salt basal medium as optimized by Tharwat [9]. The medium consisted of the following components (g/L): fibrin, 2; NH_4NO_3, 0.05; $KHPO_4$, 1.0 and $(NH_4)_2SO_4$, 0.5. The pH of the medium was adjusted to 6.5 at 35 ºC and the isolates grown on it for 24 days under stationary conditions.

Assay for fibrinolytic protease activity

Fibrinolytic protease activity was performed according to the method of Greenberg [10]. The reaction mixture contained 8 mg bovine fibrin, 500 µl mycelial extract made in phosphate buffer, (0.05 M, pH.6.8) in a total volume of 1 mL. The mixture was incubated for 30 min at 37 ℃ in a water bath and stopped by adding 0.5 mL of 15 % cold trichloroacetic acid (TCA) in glass-distilled water. The mixture was centrifuged at 3,000 g for 10 min to remove precipitated fibrin. To 0.5 mL of acid soluble filtrate, 2.5 mL of 0.3M sodium hydroxide and 2.9 %w/v sodium carbonate in glass-distilled water was added, followed by 0.75 mL of Folin's phenol reagent (diluted 1:3 with glass-distilled water). The mixture was incubated for 25 min at room temperature and the color developed was read at 650 nm. The heat-killed enzyme with fibrin substrate was used as the blank. One unit of enzyme activity was calculated as the amount of enzyme which released 1 µmol of tyrosine/min under the reaction conditions.

Purification of fibrinolytic enzyme

Unless and otherwise mentioned, all the purification procedures were carried out at 4 ℃. Protein concentrations were determined

by the method of Bradford [11]. The mycelium grown on modified Czapek's dox broth with fibrin (MCDBF) medium was collected by filtering through several layers of cheese cloth. The mycelial mat was washed with 300 mL of Tris-HCl buffer (0.05 M, pH 7.4) twice and frozen at -40 ºC. Five hundred grams of frozen mycelia were thawed and homogenized with a mortar and pestle with equal volume of Tris-HCl buffer (10 mM, pH 7.4). The homogenate was collected and centrifuged at 10,000 g for 30 min in a refrigerated centrifuge (Beckman Inc, USA). The supernatant was collected in a sterile container and the proteins were precipitated with different saturations of ammonium sulphate. The precipitated proteins were collected by dissolving in 10 mL of Tris-HCl buffer (10 mM; pH 7.4) and dialyzed against same buffer for 48 h at 4 ℃. The dialyzed fraction was centrifuged at 12,000 g for 10 min to remove the insoluble materials. The clear supernatant collected was concentrated by lyophilization and stored at -20 ℃ until further use.

Purification of the enzyme was performed first with anion exchange chromatography column. For this, DEAE cellulose powder (7.0 g) was suspended in 100 mL of distilled water for 1 h. The fine particles floating on the surface were removed and the remainder was washed several times with 1M NaOH and finally washed with glass-distilled water. The activated ion exchanger was packed in a glass column (2 × 3 cm), thoroughly washed with glass-distilled water and equilibrated with Tris-HCl buffer (20 mM; pH 7.4). The proteins were adsorbed on to the column and the unbound proteins were washed with Tris-HCl buffer (20 mM; pH 7.4). The column bound proteins were eluted with the above buffer with a gradient of NaCl (0 to 1.0 M). Protein fractions (3.0 ml each) were collected using an automatic fraction collector (LKB Broma 7000 ultra fraction collector). A total number of 50 fractions were collected and they were immediately assayed for fibrinolytic protease activity as described above. The enzyme active fractions were pooled together, concentrated by lyophilization and the extent of purity was checked on SDS-PAGE after silver staining. We observed that there were some contaminating proteins along with the enzyme of interest and therefore the enzyme had to be purified using Sephadex G-150 column (2 × 100 cm) chromatorgraphy. A total of 100 fractions (5 ml each) were collected and each of them was assayed for fibrinolytic protease activity as described above. The enzyme-active fractions were pooled, concentrated and their purity again checked on SDS-PAGE.

Effect of various metal ions on the activity of purified fibrinolytic protease

The effect of metal ions such as $MgCl_2$, $ZnCl_2$, $CoCl_2$, $FeCl_2$, $CaCl_2$ and $CuSO_4$ on the enzyme activity was studied. The purified protease (10 µg/mL in 20 mM Tris-HCL buffer, pH 7.2) was incubated with bivalent cations including Mg^{2+}, Ca^{2+} Co^{2+}, Cu^{2+}, Fe^{2+}, and Zn^{2+}ions with a final concentration of 1 mM in 50 mM Tris-HCL buffer, pH 7.2 for 1 h at 37 ℃ and the residual activity was measured with 1 % casein as per the assay method described above.

Effect of various enzyme inhibitors on purified fibrinolytic protease activity

Two milliliters of the purified protease enzyme at 5 µg/mL was pre-incubated with an equal volume of enzyme inhibitors such as PMSF, Leupetin, 1, 10-phenanthroline, TLCK, TPCK, EDTA and APMSF at two different concentrations i.e., 1 mM and 5 mM at 37 ℃ for 1 h. The residual protease activity was measured with 1 % casein using the assay method as described above.

SDS-PAGE analysis for fibrinogenolytic activity of purified protease after treatment with enzyme inhibitors

The purified protease (10 µg/mL) enzyme was treated with different enzyme inhibitors like EDTA, 1,10-phenanthroline, PMSF, TLCK, phosphoamidon and cysteine at a final

concentration of 5 mM for 1 h at 37ºC. Bovine fibrinogen, the substrate, (150 µL, 1 g/L) was added to it and incubated for a further 1 h. The reaction mixtures were subjected to electrophoresis on SDS-PAGE (12 %w/v). The gel was stained with coomassie brilliant blue R-250 and examined for hydrolytic activity of the substrate.

Effect of metal ions on the reactivation of inhibitors treated protease

The purified protease (10 µg/mL) was incubated with EDTA at a final concentration of 5 mM at 37 ^{o}C for 1 h. Thereafter, $MgCl_2$, $CaCl_2$ and $ZnCl_2$ were added to the mixture at a final concentration 5 mM and the mixture was incubated for 1 h further. The substrate bovine fibrinogen (450 µL, 1g/L) was added to the reaction mixture and incubated further for 1 h at 37 ^{o}C. The entire reaction mixture was loaded into the wells of SDS-PAGE and the protein separation was carried out. The proteins were stained with coomassie brilliant blue R-250 overnight and the gel was examined after destaining. A similar experiment with 1, 10-phenanthroline (5 mM) instead of EDTA was also performed to study the reactivation of the enzyme.

Statistical analysis

All data are presented as mean ± standard error of mean (SEM). Analysis of variance was performed by using SPSS (SPSS Inc., USA). Duncan's new multiple range test was used to determine the difference of means and $p < 0.05$ was considered statistically significant.

RESULTS

Purification of fibrinolytic enzyme

The intracellular fibrinolytic protease enzyme isolated from the mycelia mat and purified is listed in Table 1. The proteins precipitated with ammonium sulfate at 80 % saturation showed relatively higher protease activity than the proteins obtained with 30 and 60 % saturation . Therefore, the proteins obtained with 80 % saturation were used for further purification on DEAE-cellulose anion exchange column.

As shown in Table 1, 12 mg enzyme was purified at 14.4-fold from 500 g of *G. lucidum* mycelium, with a yield of 44.6 %. The purity of the enzyme was checked on SDS-PAGE after silver nitrate staining. As shown in Fig 1 (lane 4), the enzyme appeared homogenous. The molecular mass of the fibrinolytic enzyme was 33 kDa.

Effect of different metal ions on purified fibrinolytic protease of *G. lucidum*

The enzyme incubated with copper sulfate lost its complete activity even at 1 mM concentration followed by $CoCl_2$ and $FeCl_2$ to the extent of 23.6 and 84.3 % respectively, at 1 mM concentration (Table 2). The inhibitory effect of $CoCl_2$ is more pronounced when the concentration is increased to 5 mM from 1

Table 1: Purification of intracellular fibrinolytic protease from *G. lucidum*

Purification step	Total protein (mg)	Total activity (units/mL)	Specific activity (units/mg)	Purification fold	Recovery
Homogenate	175	61,250	350	1	100
Ammonium sulphate precipitation (Fraction-3)	55	48,180	876	3.1	78.6
DEAE-cellulose, Anion Exchange Chromatography	22	36,452	1656.9	7.9	59.5
Sephadex G-150 column Chromotography	12	27,350	2279.1	14.5	44.6

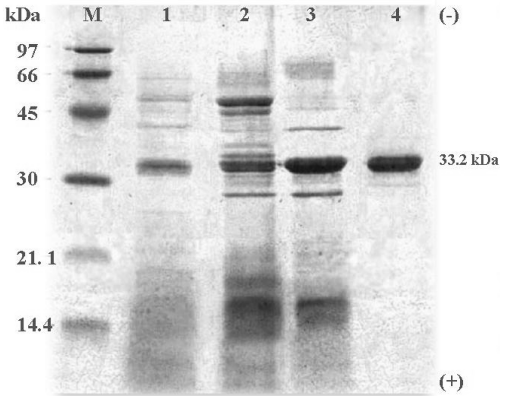

Figure 1: Molecular mass determination of purified fibrinolytic protease on SDS-PAGE under reducing conditions
Lanes: M = Protein molecular weight standards; 1 = Crude enzyme; 2 = Ammonium sulphate precipitated protein fraction-3; 3 = DEAE-cellulose column eluted proteins; 4 = Sephadex G-150 column eluted protein. (Protein molecular mass standards: 97 kDa:phosphorylase; 66 kDa: bovine serum albumin; 45 kDa: ovalbumin; 30 kDa: carbonic anhydrase; 20.1 kDa: soybean inhibitor and 14.4kDa: α-lactalbumin).

mM. On the other hand metal ions such as $CaCl_2$, $MgCl_2$ and $ZnCl_2$ enhanced the enzyme activity and the activity increase observed was 35.2, 18.3 and 8.3 %, respectively, at 1 mM concentration. There was a decrease in the enhancement of the enzyme activity when the concentration of $CaCl_2$ increased from 1mM to 5 mM.

Effect of different inhibitors on purified fibrinolytic protease of *G. lucidum*

The purified enzyme lost its complete activity when the enzyme was incubated with 1, 10-phenonthroline, EDTA and phosphoamidon at a final concentration of 5 mM (Table 2). TLCK inhibited the enzyme to a lesser extent (13.7 %). Protease inhibitors such as leupeptin, PMSF, TPCK and APMSF did not inhibit the enzyme activity but there were marginal increase in enzyme activity in these cases when compared to the control (Table 2).

SDS-PAGE analysis for fibrinogenolytic activity of purified protease after treatment with enzyme inhibitors

As shown in Fig 2, the enzyme incubated with EDTA, 1, 10-phenanthroline, phosphoamidon and cysteine (lanes 2, 3, 6 and 7) did not hydrolyze the substrate and appeared intact as that of the control (lane F); this indicates complete inactivation of the enzyme by the inhibitors. As is evident from Fig 2 (lanes 4 and 5), the enzyme treated with PMSF and TLCK hydrolyzed the substrate, indicating the inability of these inhibitors to inhibit the enzyme activity.

Table 2: Effect of protease inhibitors on purified fibrinolytic protease of *G. lucidum*

Metal ion	Residual protease activity (%)	
	1 mM	***5 mM***
Control	100±100	100±100
$ZnCl_2$	108.3±13.0	98.0±9.8
$MgCl_2$	118.3±14.2	102.0±9.2
$FeCl_2$	85.7±9.4	81.0±9.7
$CuSO_4$	No activity	No activity
$CoCl_2$	76.4±9.9	16.0±1.6
$CaCl_2$	135.2±16.2	125.0±16.3
PMSF	-	102±12.2
PT	-	No activity
TLCK	-	86.3±7.8
TPCK	-	101.0±9.1
EDTA	-	No activity
APMSF	-	107.0±8.6
PP	-	No activity
*Leupeptin	-	104.0±11.4

- *2mM final concentration; PT = 1,10-phenanthroline; PP = phosphoamidon, EDTA = Ethylene dimethyl trichloro aectic acid: PMSF = phenylmethyl sulfonyl fluoride: AMPSF = amdino phenylmethyl sulfonyl fluoride: TLCK = Tosyl-L-lysine chloromethyl ketone: TPCK = tosyl-L-phenylalanine chloromethyl ketone*

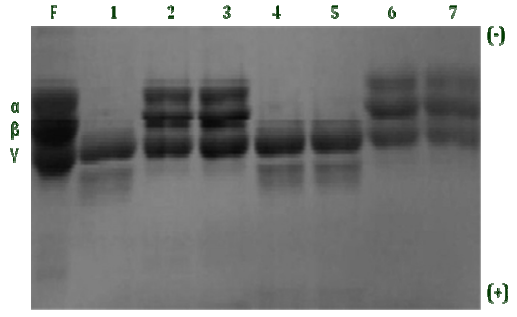

Figure 2: SDS-PAGE analysis of fibrinogenolytic activity of purified fibrinolytic protease after treatment with protease inhibitors
Lanes: F = Fibrinogen; 1 = Protease + fibrinogen; 2 = Protease + 5mM EDTA + fibrinogen; 3 = Protease + 5 mM 1,10-phenanthroline + fibrinogen; 4 = Protease + 5 mM PMSF + fibrinogen; 5 = Protease + 5 mM TLCK + fibrinogen; 6 = Protease + 5 mM phosphoamidon + fibrinogen; 7 = Protease + 5mM cysteine + fibrinogen

Effect of metal ions on the reactivation of inhibitor-treated protease

In order to verify if the enzyme inhibition by EDTA, 1, 10-phenanthroline, phosphoamidon and cysteine as observed above (Fig 2) is permanent or the activity could be restored by metal ions, the inhibited enzyme was treated with metal ions. As shown in Fig 3, addition of $ZnCl_2$, $MgCl_2$ and $CaCl_2$ to the enzyme treated with EDTA reactivated enzyme activity (Fig 3: lanes 3, 4 and 5). Similarly the enzyme inhibition by 1, 10-phenanthroline was restored by the addition of $CaCl_2$ (Figure 3: lanes 7 and 8).

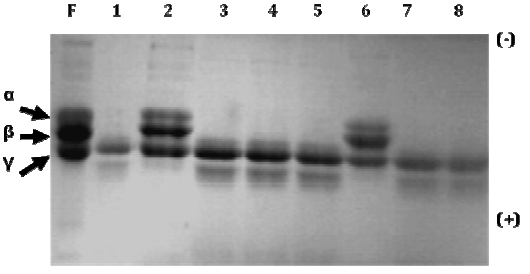

Figure 3: SDS-PAGE analysis of enzyme reactivation by metal ions after treatment with enzyme inhibitors
Lanes: F = Fibrinogen; 1 = Protease +fibrinogen; 2 = Protease +1mM EDTA+fibrinogen; 3 = Protease+1mMEDTA+$ZnCl_2$+fibrinogen; 4=Protease +1mM EDTA+$MgCl_2$+fibrinogen; 5= Protease +1mM EDTA+$CaCl_2$+fibrinogen; 6 = Protease +1mM 1,10-phenanthroline +fibrinogen; 7 = Protease +1mM 1,10-phenanthroline+1mM $CaCl_2$+fibrinogen; 8 = Protease +1mM 1,10-phenanthroline+5mM $CaCl_2$+fibrinogen

DISCUSSION

An attempt has been made in the present study to evaluate the inhibitory activity of different metal ions and some commercial protease inhibitors on the activity of an intracellular fibrinolytic protease isolated and purified from the mycelium of *G. lucidum*. As seen in Fig. 1, lane 4, the purified enzyme appeared as a single homogenous band on SDS-PAGE after silver nitrate staining and the purity of the enzyme was thus confirmed. The molecular mass of the purified enzyme was 33.2 kDa. The fibrinolytic proteases isolated from the culture filtrates of *Aspergillus fumigatus,* 33 kDa [12], exactly correspond to the molecular mass of fibrinolytic protease purified from *G. lucidum* in this study.

$CuSO_4$ completely inactivated the enzyme at all the concentrations tested followed by $CoCl_2$ which inhibited the enzyme activity to the extent of 23 and 84 % at 1 and 5 mM concentrations, respectively, compared with control. On the other hand, addition of $CaCl_2$, $MgCl_2$ and $ZnCl_2$ to the enzyme enhanced activity and the enhancement was more pronounced with $CaCl_2$. A fibrinolytic protease isolated from *Tenodera sinensis* has been shown to be sensitive to $CuSO_4$ and its activity was enhanced when the enzyme was inbubated with metal ions like Ca^{2+}, Mg^{2+} and Zn^{2+} [13].

Lee *et al*. [14] have found that the fibrinolytic enzyme purified from *Armillaria mellea* was inhibited by Cu^{2+} and Co^{2+}, but enhanced by the addition of Ca^{2+} and Mg^{2+} ions. In contrast, the fibrinolytic proteases isolated from *B. subtilis* and *B. polymxa* were not inhibited by Cu^{2+} [15], whereas the fibrinolytic proteases of A*spergillus ochraceus* HP-19 were completely resistant to the action of heavy metals, and their activities were

stabilized by the addition of Ca^{2+} ion [16]. The activity of fibrinolytic protease was recovered in the order of effectiveness by Zn^{2+}, Co^{2+} and Cu^{2+}, but not by Ca^{2+} and Mg^{2+} [17]. The protease of *Oidiodendron flavum* has been found to be enhanced by the addition of Mg^{2+} and Ca^{2+}, but was inhibited by Co^{2+} and Zn^{2+} [15].

The purified fibrinolytic protease of *G. lucidum* was highly sensitive to EDTA, 1, 10-phenanthroline and phosphoamidon and insensitive to leupeptin, PMSF, TPCK, and APMSF. The sensitivity of the purified protease to EDTA clearly shows that it could be a metalloprotease. A similar observation had been made by some authors previously [14] with the same organism; however, the molecular mass of the enzyme was estimated to be 54 kDa, which clearly indicates that the enzyme purified in this study is different from the one reported earlier. The fibrinolytic proteases of the wild mushroom, *Tricholoma saponaceum*, and the cultivated *Pleurotus ostreatus* were sensitive to EDTA and 1, 10 phenanthroline [18]. Most of the α-fibrinogenases found in snake venom are metalloproteinases and they are sensitive to the action of EDTA [19]. The metalloproteinase nature of fibrolase has been confirmed by the observation that fibrolase activity is completely and rapidly inhibited by the addition of EDTA and 1,10-phenanthroline [20].

CONCLUSION

The results obtained in the present study clearly show that copper sulfate completely inhibited the enzyme activity while Ca^{2+}, Mg^{2+} and Zn^{2+} ions enhanced the activity. The purified protease was very sensitive to protease inhibitors such as EDTA, 1, 10-phenanthroline and phosphoamidon which inhibited the enzyme completely and insensitive to TPCK, leupeptin, PMSF, and APMSF. The sensitivity of this enzyme to EDTA suggests that it might be a metalloprotease.

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