

## Research Article

# A Novel Aspartic Protease with HIV-1 Reverse Transcriptase Inhibitory Activity from Fresh Fruiting Bodies of the Wild Mushroom *Xylaria hypoxylon*

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A novel aspartic protease with HIV-1 RT inhibitory activity was isolated and characterized from fruiting bodies of the wild mushroom *Xylaria hypoxylon*. The purification protocol comprised distilled water homogenization and extraction step, three ion exchange chromatographic steps (on DEAE-cellulose, Q-Sepharose, and CM-cellulose in succession), and final purification was by FPLC on Superdex 75. The protease was adsorbed on all the three ion exchangers. It was a monomeric protein with a molecular mass of 43 kDa as estimated by SDS-PAGE and FPLC. Its N-terminal amino acid sequence was HYTELLSQVV, which exhibited no sequence homology to other proteases reported. The activity of the protease was adversely affected by Pepstatin A, indicating that it is an aspartic protease. The protease activity was maximal or nearly so in the pH range 6–8 and in the temperature range 35–60°C. The purified enzyme exhibited HIV-1 RT inhibitory activity with an IC<sub>50</sub> value of 8.3 μM, but was devoid of antifungal, ribonuclease, and hemagglutinating activities.

## 1. Introduction

Mushrooms produce spectacular diversity of biologically active biomolecules encompassing laccase, lectins, nucleases, proteases, antifungal proteins, ribosome inactivating proteins, and polysaccharides, polysaccharide—peptides and polysaccharide—protein complexes [1]. Many of these proteinaceous and nonproteinaceous molecules manifest potentially worthwhile activities including antitumor, HIV-1 reverse transcriptase inhibitory, and immunomodulatory activities [1, 2]. A variety of mushroom constituents are known to have health promoting activities and therapeutic

potential. Some of them are under clinical investigations [3, 4]. Many mushrooms have a good taste and are highly nutritious. Thus mushrooms are popular in the diet of many people all over the world.

Proteases catalyze degradation with high (e.g., trypsin) or low (e.g., subtilisin) specificity. [5, 6]. They can be produced in sizeable quantities by employing microbial approaches. The requirements of brewing, dairy, meat, leather, detergent, and photographic industries make proteases useful and commercial [7]. The sale of industrial enzymes, a significant percentage of which is used for detergents, is a business involving millions of dollars [8]. Proteases

have been reported from a number of mushrooms, including *Agaricus bisporus* [9], *Armillariella mellea* [10], *Cordyceps sinensis* [11], *Flammulina velutipes* [12], *Grifola frondosa* [13], *Helvella lacunosa* [14], *Lyophyllum cinerascens* [15], *Pleurotus eryngii* [16], *Pleurotus ostreatus* [17], *Pleurotus citrinopileatus* [18], and *Tricholoma saponaceum* [19].

*Xylaria hypoxylon*, commonly known as candlestick fungus, carbon antlers, or stag's horn fungus, is an inedible mushroom belonging to the genus *Xylaria* [20]. In China, *X. hypoxylon* is a kind of competitor fungus found in the cultivation of straw mushroom (*Volvariella volvacea*). A variety of bioactive compounds have been isolated from the fungus. Two tetralone derivatives xylariol A and B, and two  $\alpha$ -pyrone derivatives xylarone and 8,9-dehydroxylarone possessing cytotoxic activities were isolated from the culture broth of *X. hypoxylon* [21, 22]. Sex cytochalasins binding to actin in muscle tissue, a xylose-specific lectin with antiproliferative, and antimitogenic activities have also been found in the fungus [23, 24]. In view of the importance of proteases and the differences in characteristics of proteases from different sources and the dearth of information on protease from wild mushrooms, the present study was undertaken to purify and characterize a protease from the wild mushroom *X. hypoxylon*.

## 2. Materials and Methods

**2.1. Fungal Material and Reagents.** Fruiting bodies of *X. hypoxylon* were purchased from a company specializing on straw mushroom in Beijing and identified by Institute of Microbiology, Chinese Academy of Sciences. The sources of other biochemical and chemical reagents used in this work are as follows: DEAE-cellulose, CM-cellulose, Coomassie brilliant blue R-250, glycine, casein, trypsin, and yeast tRNA, were obtained from Sigma. Q-Sepharose, Superdex 75, molecular mass standards, and AKTA Purifier were purchased from GE Healthcare (USA). All other reagents were of reagent grade.

**2.2. Isolation of Protease.** A water extract of the fruiting bodies of *X. hypoxylon* (500 g) was prepared by homogenization in distilled water (4 mL/g). Following centrifugation of the homogenate at 12000 g for 20 minutes, Tris-HCl buffer (pH 7.2) was added to the supernatant obtained until the concentration of Tris was 10 mM. Ion exchange chromatography of the supernatant on a 5 × 20 cm column of DEAE-cellulose was then carried out in 10 mM Tris-HCl buffer (pH 7.2). After removal of the flow-through fraction (D1), the column was eluted stepwise with 0.2 M NaCl and then with 1 M NaCl in the starting buffer to yield fractions D2 and D3, respectively. Fraction D3 was dialyzed, lyophilized, and chromatographed on a Q-Sepharose column (2.5 × 20 cm) in 10 mM Tris-HCl buffer (pH 7.0). When all the unadsorbed proteins (collected as fraction Q1) had been eluted, the column was eluted with a linear concentration (0–1 M) gradient of NaCl added to 10 mM Tris-HCl buffer (pH 7.2). The second and most strongly adsorbed fraction, Q3, was dialyzed, lyophilized, and then applied to a 2.5 × 20 cm

column of CM-cellulose. The column was eluted with 10 mM NH<sub>4</sub>OAc buffer (pH 4.5) until all the unadsorbed proteins had been eluted and collected as fraction CM1. Adsorbed proteins were desorbed with a linear concentration (0–1 M) gradient of NaCl in 10 mM NH<sub>4</sub>OAc buffer (pH 4.5) to yield fractions CM2 and CM3. Final purification was conducted by FPLC-gel filtration of fraction CM2 on a Superdex 75 HR 10/30 column in 0.2 M NH<sub>4</sub>HCO<sub>3</sub> buffer (pH 8.5) using an AKTA Purifier. The second eluted peak represented purified protease. All the purification steps were carried out at 4°C.

**2.3. Molecular Mass Determination by SDS-PAGE and by FPLC-Gel Filtration.** SDS-PAGE was assayed using the protocol of Laemmli and Favre [25], using a 12% resolving gel and a 5% stacking gel. At the end of electrophoresis, the gel was dyed with 0.1% Coomassie brilliant blue R-250. FPLC-gel filtration was carried out using a Superdex 75 HR 10/30 column which had been calibrated with the undermentioned molecular mass standards [26]. The molecular mass of the protein was determined by comparison of the elution volume with those of molecular mass standards including blue dextran (to determine void volume), phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), soybean trypsin inhibitor (20 kDa), and bovine  $\alpha$ -Lactalbumin (14.4 kDa).

**2.4. Analysis of N-Terminal Amino Acid Sequences.** Amino acid sequence analysis was carried out using an HP G1000A Edman degradation unit and an HP1000 HPLC system [27].

**2.5. Assay for Protease Activity.** In this assay, an improved method of Satake et al. [28] was used. In brief, a casein solution, which was used as substrate, was freshly prepared as follows: 0.1 g casein was added into 10 mL Mes buffer (200 mM, pH 7.0). Subsequently, the solution was incubated at 60°C for 30 min. Following centrifugation at 12000 g for 20 minutes, the precipitate was removed and the resulting solution could be used as the protease substrate. The test sample or trypsin solution (as positive control) (20  $\mu$ L) was mixed with the above casein solution (180  $\mu$ L) and then incubated at 37°C for 15 min. Subsequently, 400  $\mu$ L of 5% trichloroacetic acid (TCA) was added for ending the enzymatic reaction. The reaction mixture was then cooled to the room temperature before centrifugation at 10000 g for 15 min. The absorbance of the supernatant was read at 280 nm against water as blank using a UV-spectrophotometer. Protease activity was calculated based on the activity of trypsin (7900 BAEE units/mg according to Sigma) in the protease assay using casein as substrate [29].

**2.6. Optimal pH and Temperature of Purified Protease.** Protease activity was tested over the pH range (pH 3–9) and temperature range (20°C–100°C). Casein in different buffers including 0.1 M NaOAc (pH 3–5), 0.1 M Mes (pH 5–7), and 0.1 M Hepes (pH 7–9) were used to determine the optimal pH value. To determine the optimal temperature, the reaction mixture was incubated at 20°C, 30°C, 37°C, 45°C,

50°C, 60°C, 70°C, 80°C, and 100°C in 0.1 M Mes buffer (pH 7.0) for 30 min [18].

**2.7. Assay for Mechanistic Class.** In this assay, the purified protease was exposed to the following inhibitors: phenylmethylsulfonyl fluoride (PMSE, 1.0 mM), ethylenediaminetetraacetic acid disodium salt (EDTA, 1.0 mM), pepstatin A (0.2 mM), and lima bean trypsin inhibitor (0.25 mM) for 30 min at room temperature. Residual enzyme activity was measured using the standard assay above. Control, in which inhibitors were substituted by Mes buffer (pH 7.0), was taken as 100% [26].

**2.8. Assay for HIV-1 Reverse Transcriptase Inhibitory Activity.** The assay for HIV-1 reverse transcriptase inhibitory activity was carried out using the assay kit from Boehringer Mannheim (Germany) and the reaction protocol of Zhao et al. [30].

**2.9. Assay for Antifungal Activity.** The assay for antifungal activity toward *Fusarium oxysporum*, *Rhizoctonia cerealis*, *Rhizoctonia solani*, and *Sclerotinia sclerotiorum* was carried out using the method of Lam and Ng [31].

**2.10. Assay for Ribonuclease Activity.** The ribonuclease activity of the purified protease was assayed following the method of Mock et al. [32] and using yeast tRNA as substrate.

**2.11. Assay for Hemagglutinating Activity.** The assay for hemagglutinating activity was measured with a 2% suspension of rabbit red cells in phosphate-buffered saline (pH 7.2) at 20°C with the method of Zhang et al. [33].

### 3. Results

**3.1. Isolation of Protease.** After ion exchange chromatography of the fruiting body extract on DEAE-cellulose, protease activity resided only in fraction D3 which was most strongly adsorbed on the column. Both fractions D1 and D2 were devoid of protease activity (Table 1). Protease activity was again enriched in the most strongly adsorbed fraction, Q3, derived by ion exchange chromatography of fraction D3 on Q-Sepharose. The unadsorbed fraction Q1 lacked protease activity and the adsorbed fraction Q2 exhibited very low protease activity (Table 1). Further purification of the unadsorbed fraction Q3 on CM-cellulose allowed separation of a small unadsorbed peak from two adsorbed peaks (Figure 1). No protease activity was detected in the unadsorbed fraction CM1. The first adsorbed fraction (CM2) had much higher protease activity than the following adsorbed fraction (CM3) (Table 1). CM2 was resolved into two peaks of similar sizes upon gel filtration on Superdex 75 (Figure 2). The first fraction, SU1, was without protease activity. Protease activity was confined to the second fraction, SU2 (Table 1). SU2 appeared as a single band with a molecular mass of 43 kDa in SDS-PAGE (Figure 3) and as a single peak with the same molecular mass upon rechromatography on Superdex 75

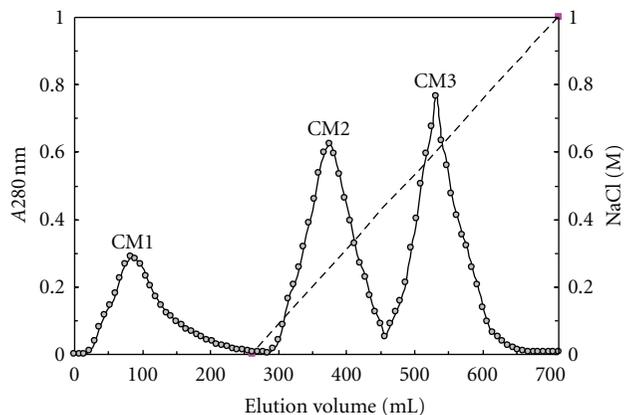


FIGURE 1: Ion exchange chromatography on CM-cellulose. Sample: fraction Q3. Fraction size: 8 mL. Flow rate: 2 mL/min. Dotted line across right-hand side of chromatogram indicates linear NaCl concentration (0-1 M) gradient used to CM2 and CM3.

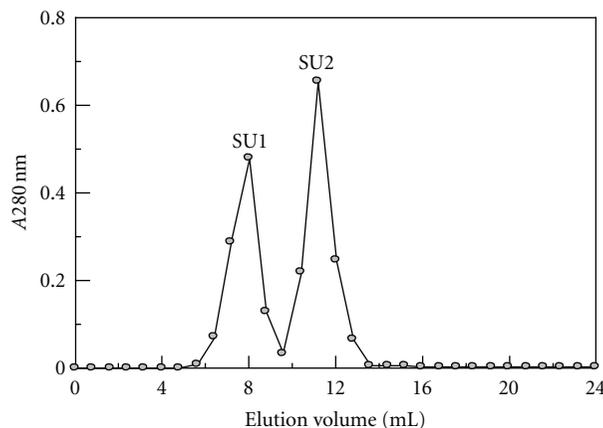


FIGURE 2: FPLC-gel filtration on Superdex 75 HR 10/30 column. Sample: fraction CM2. Eluent: 0.2 M  $\text{NH}_4\text{HCO}_3$  buffer (pH 8.5). Fraction size: 0.8 mL. Flow rate: 0.4 mL/min.

(data not shown). The enzyme was purified 74.1-fold from the crude extract with 43.6% yield. The purified protease exhibited an activity of 459.3 U/mg (Table 1).

**3.2. Characterization of Isolated Protease.** The N-terminal amino acid sequence of purified *X. hypoxylon* protease was HYTELLSQVV. A comparison of characteristics of *X. hypoxylon* and other fungal proteases is listed in Table 2. The protease was strongly inhibited by Pepstatin A, but not significantly affected by PMSE, EDTA, and Trypsin inhibitor (Table 3). The protease activity increased steadily as the pH was raised from 3.0 to 6.0 and then remained high when the pH was further raised to 8.0. There was an approximately 12% decrease in activity as the pH reached 9.0 (Figure 4). The protease activity escalated as the ambient temperature was raised from 20°C to 40°C. There was very little change in activity between 40°C and 60°C. As the temperature was raised to 100°C, there was a quick fall in activity. However, about 30% of the maximal activity was remained at 100°C

TABLE 1: Yields and protease activities of various chromatographic fractions of *Xylaria hypoxylon* (from 500 g fresh fruiting bodies. Assay conditions: 37°C/15 min, 0.1 M Mes buffer, pH 7.0).

Fraction	Yield (mg)	Protease activity (u/mg)	Total activity (u)	Recovery of activity (%)	Purification fold
<b>Extract</b>	<b>2038</b>	<b>6.2</b>	<b>12636</b>	<b>100</b>	<b>1</b>
D1	506	<0.5	—	—	—
D2	405	<0.5	—	—	—
<b>D3</b>	<b>534</b>	<b>20.3</b>	<b>10840</b>	<b>85.8</b>	<b>3.3</b>
Q1	68	<0.5	—	—	—
Q2	163	1.9	310	2.5	0.3
<b>Q3</b>	<b>148</b>	<b>57.9</b>	<b>8569</b>	<b>67.8</b>	<b>9.3</b>
CM1	30	<0.5	—	—	—
<b>CM2</b>	<b>31</b>	<b>219.4</b>	<b>6801</b>	<b>53.8</b>	<b>35.4</b>
CM3	41	2.8	115	0.9	0.5
SU1	9	<0.5	—	—	—
<b>SU2</b>	<b>12</b>	<b>459.3</b>	<b>5512</b>	<b>43.6</b>	<b>74.1</b>

—: no protease activity observed.

Protease-enriched fractions are highlighted in boldface.

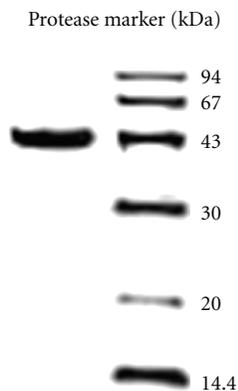


FIGURE 3: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis results. Left lane: purified protease (fraction SU2, 10 µg). Right lane: molecular mass markers, from top downward: phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), and  $\alpha$ -lactalbumin (14.4 kDa).

(Figure 5). At a protease concentration of 1 µM, 5 µM, 15 µM, and 25 µM, 17.5%, 42.3%, 65.6%, and 79.8% inhibition, respectively of HIV-1 reverse transcriptase were observed (Figure 6). The IC<sub>50</sub> value was estimated to be 8.3 µM. It did not exhibit antifungal, ribonuclease, and hemagglutinating activities (data not shown).

#### 4. Discussion

The present study constitutes the first report on the purification of a protease from *Xylaria hypoxylon*. A comparison of chromatography behavior of *X. hypoxylon* and other fungal proteases is listed in Table 2. *X. hypoxylon* protease, just like *H. lacunose* protease, adsorbs on DEAE-, CM-cellulose,

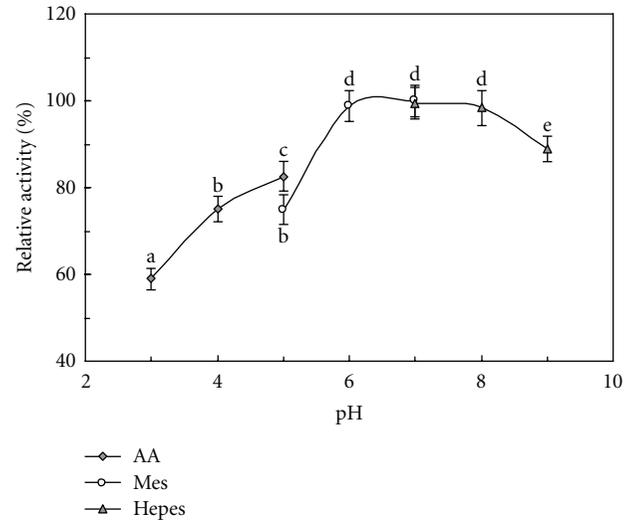


FIGURE 4: Effect of pH on activity of the purified protease. Results are presented as mean  $\pm$  SD ( $n = 3$ ). Different letters (e.g., a, b, c, and d) next to the data points indicate statistically significant difference ( $P < 0.05$ ) when the data are analyzed by analysis of variance followed by Duncan's multiple range test.

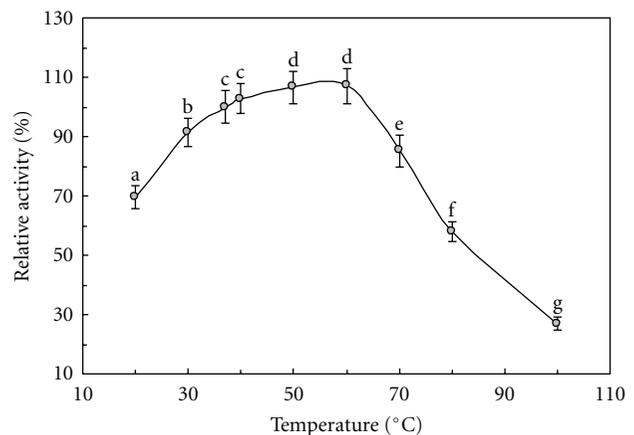


FIGURE 5: Effect of temperature on activity of the purified protease. Results are presented as mean  $\pm$  SD ( $n = 3$ ). Different letters (e.g., a, b, c, and d) next to the data points indicate statistically significant difference ( $P < 0.05$ ) when the data are analyzed by analysis of variance followed by Duncan's multiple range test.

and Q-Sepharose [14]. A protease from *P. citrinopileatus* unadsorbs on DEAE-cellulose and Q-Sepharose, but adsorbs on CM-cellulose [18]. So far a few ascomycetous proteases from genus *Aspergillus*, *Cordyceps*, *Helvella*, and *Xylaria* have been reported, and only *C. sinensis*, *H. lacunose* proteases, and the present one are from mushroom species. Compared with each other, the three ascomycetous proteases displayed different characters. *X. hypoxylon* and *H. lacunose* proteases manifests a close temperature optima (60°C and 65°C, resp.), while *C. sinensis* protease possesses a quite low optimal temperature of 30°C. *X. hypoxylon* and *C. sinensis* proteases exhibit a same molecular mass of 43 kDa which is higher than

TABLE 2: Comparison of characteristics of *Xylaria hypoxylon* protease with other fungal proteases.

Species	Phylum	N-terminal sequence	Chromatography behavior	Molecular mass (kDa)	pH optimum	Temperature optimum (°C)
<i>Xylaria hypoxylon</i> (This study)	Ascomycota	1 HYTEL LSQVV 10	Adsorbed on DEAE-, CM-cellulose, and Q-Sepharose	43	6–8	60
<i>Aspergillus clavatus</i> [34]	Ascomycota	1 ALTTQ SGAPW GLGSI 15	Adsorbed on CM-Sepharose	32	8.5	50
<i>Aspergillus nidulans</i> [35]	Ascomycota	—	—	19	5.5	65
<i>Cordyceps sinensis</i> [11]	Ascomycota	X DNLMR AVGAL LR X	Adsorbed on HiTrap Q XL	43	9.5	30
<i>Helvella lacunosa</i> [14]	Ascomycota	1 ANVVQ WPVPC 10	Adsorbed on DEAE-, CM-cellulose, and Q-Sepharose	33.5	11	65
<i>Agaricus bisporus</i> [9, 36]	Basidiomycota	1 MHFSL SFATL ALLVA 15	Adsorbed on DEAE-, and CM-cellulose	27	6.5–11.5	—
<i>Armillariella mellea</i> [10]	Basidiomycota	1 XXYNG XT <sup>u</sup> SR QTTLV 15	Adsorbed on DEAE-cellulose	55	7	55
<i>Coprinus</i> 7 N [37]	Basidiomycota	—	Adsorbed on DEAE-Sepharose	33	8.5	37
<i>Grifola frondosa</i> [38]	Basidiomycota	1 AQTNA PWGLA 10	—	20	9–10	—
<i>Hypsizigus marmoreus</i> [26]	Basidiomycota	1 VTQTN APWGL ARLSQ 15	Adsorbed on CM-cellulose; Unadsorbed on DEAE-cellulose	28	7.5	50
<i>Pleurotus citrinopileatus</i> [18]	Basidiomycota	1 VCQCN APWGL 10	Adsorbed on CM-cellulose; Unadsorbed on DEAE-cellulose and Q-Sepharose	28	10	50
<i>Pleurotus eryngii</i> [16]	Basidiomycota	1 GPQFP EA 7	Adsorbed on Affi-gel Blue gel and CM-Sepharose; Unadsorbed on DEAE-cellulose	11.5	5.0	45

—: no data available. Identical corresponding amino acid residues are underscored.

that of *H. lacunose* (33.5 kDa). *C. sinensis* and *H. lacunose* proteases are both alkaline proteases (with optimal pH of 9.5 and 11, resp.), while *X. hypoxylon* proteases has a fairly stable optimal activity in a pH range of 6–8.

Microbial proteases display a range of molecular masses from 18 kDa to 126 kDa [39]. *X. hypoxylon* protease exhibits

an intermediate molecular mass (43 kDa), which is the same as *C. sinensis* protease, but higher than those of proteases from *P. eryngii* (11.5 kDa) [16], *T. saponaceum* (about 18 kDa) [19], *A. mellea* (18.5 kDa) [10], *G. frondosa* (20 kDa) [38], *P. citrinopileatus* (28 kDa) [18], and *H. lacunose* (33.5 kDa) [14] and lower than *P. ostreatus* (75 kDa) [17].

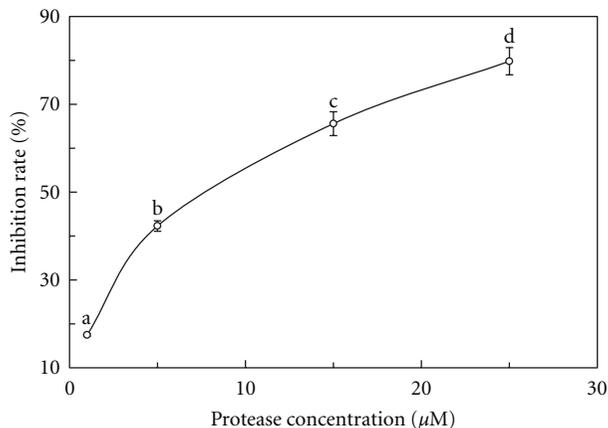


FIGURE 6: Inhibitory activity of purified protease on HIV-1 RT activity. Results represent mean  $\pm$  SD ( $n = 3$ ). The  $IC_{50}$  value toward HIV-1 RT was 1 estimated to be  $8.3 \mu\text{M}$ . Different letters (a, b, c, etc.) next to the data points indicate statistically significant difference ( $P < 0.05$ ) when the data are analyzed by analysis of variance followed by Duncan's multiple range test.

TABLE 3: Effects of protease inhibitors on *Xylaria hypoxylon* protease activity.

Protease inhibitor	Activity remaining (%)
PMSF (1.0 mM)	100.44 $\pm$ 2.44 <sup>a</sup>
EDTA (1.0 mM)	94.23 $\pm$ 0.84 <sup>a</sup>
Pepstatin A (0.2 mM)	63.51 $\pm$ 0.84 <sup>b</sup>
Lima bean trypsin inhibitor (0.25 mM)	102.09 $\pm$ 0.99 <sup>a</sup>

Activity of the control, in which inhibitors were substituted by Mes buffer (pH 7.0), was regarded as 100%. The results shown are mean  $\pm$  SD ( $n = 3$ ). Different superscripts (e.g., a, b, c) indicate a statistically significant difference ( $P < 0.05$ ) when the data corresponding to different protease inhibitors are analyzed by analysis of variance followed by Duncan's multiple range test.

Its N-terminal sequence is largely different from previously reported fungal protease sequences, suggesting that it is a new protein.

Among the protease inhibitors tested, PMST, EDTA, and Trypsin inhibitor have no significant effect on the protease activity, suggesting that it is neither a serine protease, nor a metalloprotease. The activity of the protease was adversely affected by Pepstatin A, indicating that it is an aspartic protease. To date, many serine proteases have been isolated from mushrooms, such as *A. bisporus* [40], *Cordyceps sobolifera* [41], *H. lacunose* [14], and *H. marmoreus* [26]. Other mushroom proteases are metalloproteases, including *A. mellea* [10], *G. frondosa* [38], and *T. saponaceum* [19]. Only a few mushroom proteases are belonging to aspartic proteases, such as *P. eryngii* protease [16]. The present report of *X. hypoxylon* protease thus adds to the literature.

*X. hypoxylon* protease is relatively thermostable because its activity remains within the range of 65%–100% of its maximal activity at 60°C as the temperature varies from 20°C to 70°C. At a high temperature of 80°C, a significant

portion (about 50%) of enzyme activity remains and at 100°C there is still 30% residual activity. *A. mellea* protease is stable up to 55°C, but its activity rapidly declines at higher temperatures [10]. The optimal temperature of *X. hypoxylon* protease is very close to that of *H. lacunose* protease (65°C) [14], and higher than that of *P. eryngii* (45°C) [16], *A. clavatus* (50°C) [34], *H. marmoreus* (50°C) [26], and *T. saponaceum* (55°C) [19].

The protease activity remains essentially unaltered in the pH range 6–8 and 60%–100% of the maximal activity is present even of the pH is changed from 3–9. *P. eryngii* protease demonstrates an acidic pH optimum of 5.0 [16], while several proteases of other mushrooms manifest optimal pH in the alkaline range, such as proteases from *C. sinensis* (pH 9.5) [11], *P. citrinopileatus* (pH 10) [18], and *H. lacunose* (pH 11) [14]. Protease from *H. marmoreus* exhibits a pH optimum of 7.5. Rapid decline in activity is noted, when the pH value is lower or higher than pH 7.5 [26]. Hence, the isolated protease from *X. hypoxylon* appears to be relatively stable with regard to variations in ambient pH and temperature, compared with *H. marmoreus* protease.

*X. hypoxylon* protease demonstrates HIV-1 reverse transcriptase inhibitory activity, with an  $IC_{50}$  value of  $8.3 \mu\text{M}$ . Many mushroom proteins, such as lectins, ribonucleases, and proteases, manifest inhibitory activity toward HIV-1 reverse transcriptase. The  $IC_{50}$  values of *I. umbrinella* lectin, *L. shimeji* ribonuclease, *P. eryngii* protease, *H. erinaceum* lectin, are  $4.7 \mu\text{M}$ ,  $7.2 \mu\text{M}$ ,  $30 \mu\text{M}$ , and  $31.7 \mu\text{M}$ , respectively [16, 30, 42, 43]. *X. hypoxylon* protease is the first ascomycetous protease reported manifesting HIV-1 RT inhibitory activity. Up till now, only a basidiomycetous protease from *P. eryngii* has been reported to inhibit HIV-1 RT. Inhibitory activity of the purified protease is higher than those of *P. eryngii* protease and *H. erinaceum* lectin, but lower than those of *I. umbrinella* lectin and *L. shimeji* ribonuclease. The inactivity of *X. hypoxylon* protease in hydrolyzing tRNA, inhibiting fungal growth and agglutinating rabbit red blood cells indicates that it is free of contamination with ribonucleases, antifungal proteins, and lectins that are commonly produced by mushrooms.

In summary, *X. hypoxylon* protease is a unique enzyme with a distinctive N-terminal sequence, relatively high thermostability and the first report of HIV-1 reverse transcriptase inhibitory activity from Ascomycota mushrooms.

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