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Cordysobin, a novel alkaline serine protease with HIV-1 reverse transcriptase inhibitory activity from the medicinal mushroom *Cordyceps sobolifera*

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A novel serine protease, designated as cordysobin, was purified from dried fruiting bodies of the mushroom *Cordyceps sobolifera*. The isolation procedure utilized ion exchange chromatography on DEAE-cellulose and SP-Sephadex followed by gel filtration on Superdex 75. The protease did not adsorb on DEAE-cellulose but bound to SP-Sephadex. In sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the protease resolved as a single band with an apparent molecular mass of 31 kDa. Its optimal pH was 10.0, and the optimal temperature was 65°C. The protease displayed a K_m value of 0.41 μM and 13.44 $\mu\text{M} \cdot \text{min}^{-1}$ using Suc-Leu-Leu-Val-Tyr-MCA as substrate at pH 10.0 and 37°C. Protease activity was enhanced by the Fe^{2+} ion at low concentration range of 1.25–10 mM and was strongly inhibited by Hg^{2+} up to 1.25 mM. The protease was strongly inhibited by chymostatin and phenylmethylsulfonyl fluoride (PMSF), suggesting that it is a serine protease. It manifested significant inhibitory activity toward HIV-1 reverse transcriptase (RT) with an IC_{50} value of $8.2 \times 10^{-3} \mu\text{M}$, which is the highest anti-HIV-1 RT activity of reported mushroom proteins.

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[Key words: Alkaline serine protease; Fruiting bodies; Mushroom; *Cordyceps sobolifera*; Purification; HIV-1 reverse transcriptase]

Mushrooms form an important part of the diet in many countries due to their delicious taste and nutritive value. A variety of compounds with important biological activities have been purified from mushrooms including hemolysins (1), antifungal proteins (2), laccases (3), proteases (4), RNases (5), hydrophobins (6) and lectins (7–8). Some of these compounds have exploitable potential or have proven clinical efficacy (9–10). Proteases are a class of enzymes that occupy a vital position with respect to their applications in industrial fields, i.e., in the detergent, food, pharmaceutical, leather, and silk industries and for the recovery of silver from used X-ray films (11–15). They have been isolated from different organisms including animals (16–17), plants (18–20), and microorganisms. In mushrooms, proteases have been isolated from *Agaricus bisporus* (21–22), *Armillariella mellea* (23–24), *Flammulina velutipes* (25), *Grifola frondosa* (26), *Helvella lacunosa* (4), *Lyophyllum cinerascens* (27), *Pleurotus eryngii* (28), *Pleurotus ostreatus* (29), *Pleurotus citrinopileatus* (30) and *Tricholoma saponaceum* (31).

The mushroom *Cordyceps sobolifera*, a well-known and prized traditional Chinese medicine, is an entomogenous fungal species that is parasitic on wing-less cicada nymphs. It was reported to produce inhibitors of the phenoloxidase-activating systems of insects and to secrete various enzymes that facilitate its infection of and proliferation

in parasites (32–33). The biological functions of its chemical constituents have been reported, including myriocin (34–36), nucleosides (37), sterols (38), and polysaccharides (39–40). However, no protease has yet been isolated from *C. sobolifera*. In the present study, we purified a novel protease from *C. sobolifera*, designated as cordysobin, which exhibits significant HIV-1 reverse transcriptase inhibitory activity. Other characteristics, including molecular mass, N-terminal sequence, optimal temperature, and optimal pH, were also investigated.

MATERIALS AND METHODS

Materials Dried fruiting bodies of *C. sobolifera* mushrooms were collected in Anhui, China. DEAE-cellulose, Pepstatin A, EDTA, PMSF, LBti, E-64, chymostatin, Elastatinal, casein sodium salt, Succinyl-Leu-Leu-Val-Tyr-4-methyl-coumaryl-7-amide (Suc-Leu-Leu-Val-Tyr-MCA) and other synthetic fluorogenic peptide substrates (MCA-substrates) were obtained from Sigma. Trypsin was purchased from Promega. SP-Sephadex and Superdex 75 were from GE Healthcare. All other chemicals were of reagent grade.

Purification of protease Dried fruiting bodies of *C. sobolifera* mushrooms (25 g) were homogenized and extracted overnight in 0.15 M NaCl at 4°C. The homogenate was centrifuged at $8000 \times g$ for 20 min, and then $(\text{NH}_4)_2\text{SO}_4$ was added to the supernatant to 70% saturation. Four hours later, the precipitate was collected by centrifugation at $6000 \times g$ for 30 min. The precipitate was dissolved and dialyzed to remove $(\text{NH}_4)_2\text{SO}_4$ before loading onto an anion-exchange column (2.5 \times 20 cm) of DEAE-cellulose pre-equilibrated with 10 mM NH_4HCO_3 buffer (pH 9.4). The column was eluted with the same buffer. After removal of the unadsorbed peak (fraction D1) containing strong protease activity, two adsorbed peaks, fractions D2 and D3 were eluted with 100 mM and 1 M NaCl, respectively, in the starting buffer. The active fraction (D1) was next subjected to a cation-exchange column (1.0 \times 15 cm) of SP-

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Sephacrose pre-equilibrated in 10 mM citric acid buffer (pH 5.0). After removal of unadsorbed proteins (fraction SP1), the column was eluted with a linear concentration gradient of 0–800 mM NaCl in 10 mM citric acid buffer (pH 5.0) to obtain adsorbed proteins (fractions SP2 and SP3). The active fraction (SP3) with protease activity was subsequently purified on a Superdex 75 HR 10/30 column in 0.15 M NH_4HCO_3 buffer (pH 8.5) by fast protein liquid chromatography (FPLC) using an AKTA Purifier (GE Healthcare). The first fraction (SU1) contained the purified protease.

Determination of molecular mass and amino acid sequence The apparent molecular mass of the native purified protease (SU1) was measured using the calibration of Superdex 75 10/30 column as described by Zhang et al. (41). The apparent molecular mass of the denatured protease was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli and Favre (42) using a 5% (w/v) stacking gel and a 15% (w/v) separating gel. The molecular mass of cordysobin was estimated using a low molecular mass calibration kit as markers consisting of phosphatase b (94 kDa), bovine serum albumin (67 kDa), egg white ovalbumin (43 kDa), bovine carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa), and bovine α -Lactalbumin (14.4 kDa).

Cordysobin was subjected to SDS-PAGE under reducing conditions and electrophoretically transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore) by the method of Matsudaira (43). After the membrane was briefly stained by Coomassie Brilliant Blue R-250, the protein band was cut out and applied to an ABI Procise 492 protein sequencer (Applied Biosystems) (44).

Mass spectrometry Cordysobin was subjected to trypsin digestion and the digestion products were desalted and concentrated. MS/MS of peptides generated by the in-gel digestion was performed by nano-ESI on a mass spectrometer (Thermo Fisher Scientific). Product ions were analyzed using an orthogonal TOF analyzer fitted with a reflector, a microchannel plate detector, and a time-to-digital converter. The data were processed using a PC running Bioworks Browser 3.3 software on the Windows NT environment. Sequence homologues were searched using BLAST (www.ncbi.nlm.nih.gov/BLAST.cgi) (45).

Assay for proteolytic activity The standard proteolytic activity against casein in protein purification was assayed according to the method of Satake et al. (46). The reaction mixture (165 μl) consisted of 140 μl of 1% (w/v) casein in 50 mM phosphate buffer (pH 7.5) and 25 μl of cordysobin solution (1.75 μg). The reaction was started by adding enzyme solution at 37°C. After incubation for 15 min, the reaction was stopped by adding 600 μl of 5% (w/v) trichloroacetic acid. The reaction mixture was kept on ice for another 10 min, then centrifuged at $12,000 \times g$ for 5 min at 4°C. The absorbance of the supernatant was read at 280 nm against water as a blank using a UV spectrophotometer. Protease activity was expressed in units, where one unit represented a 0.001 absorbance increase per minute in the supernatant per ml of reaction mixture under specified conditions (4).

Serine specific proteolytic activity against Suc-Leu-Leu-Val-Tyr-MCA in functional assays was measured according to the method of Jiang et al. (47). Appropriately 25 μl of diluted cordysobin (1 μg) was added to 450 μl of 10 mM Tris-HCl buffer, pH 8.0. The reaction was immediately initiated by the addition of 25 μl of 1 mM substrate and incubated at 37°C for 10 min. To stop the reaction, 750 μl of the stopping agent (methyl alcohol:isopropyl alcohol:distilled water = 35:30:35, v/v) was added. The fluorescence intensity of the liberated 7-amino-4-methylcoumarin (AMC) was measured by a fluorescence microplate reader (Tecan) at an excitation wavelength of 380 nm and an emission wavelength of 450 nm. One unit of enzyme activity was defined as the amount of the enzyme to release 1 nmol of AMC/min.

Effect of pH and temperature on the protease activity The optimal pH and temperature of cordysobin were measured by using Suc-Leu-Leu-Val-Tyr-MCA as substrate. For the pH assay, the activity of cordysobin was determined at the temperature of 37°C for 10 min at a pH range of 4.0–12.0 using a wide range pH buffer (6.008 g citric acid, 3.893 g KH_2PO_4 , 1.769 g boric acid, and 5.266 g barbituric acid dissolved in 1000 ml of distilled water. The pH of the reaction buffer was adjusted to range from pH 4.0 to 12.0 using 0.2 M NaOH). For the temperature profile study, the activity was assayed at a temperature range between 20°C and 90°C using 10 mM Tris-HCl buffer (pH 8.0).

Assay of the enzyme thermostability To examine the effects of temperature on the stability of cordysobin, 25 μl diluted enzyme solution (1 μg) was incubated at temperatures from 0°C to 70°C for 40 min, followed by immediate cooling in ice water and assayed using Suc-Leu-Leu-Val-Tyr-MCA as substrate. Residual activity was determined at 37°C.

Assay of enzyme kinetics Suc-Leu-Leu-Val-Tyr-MCA solutions (2 μM , 1 μM , 0.8 μM , 0.4 μM , 0.2 μM , 0.1 μM) were used as substrates. The K_m and V_{max} of the enzyme were calculated based on the Lineweaver-Burk plot constructed by plotting the reciprocal of the substrate concentration on the X-axis and the reciprocal of the enzyme reaction velocity on the Y-axis. All determinations were performed in triplicate at pH 10.0 and 37°C.

Effect of metal ions and enzyme inhibitors To examine the effects of various metal ions on cordysobin activity, the chloride salts of the metal ions were added to four reaction mixtures at final concentrations of 1.25, 2.5, 5.0, and 10 mM. After incubation at 4°C for 60 min, the protease activity using Suc-Leu-Leu-Val-Tyr-MCA as substrate was assayed as described above (30).

To determine the hydrolysis type of cordysobin, 25 μl purified protease (1 μg) was pre-incubated with the following inhibitors: Pepstatin A, ethylenediamine tetraacetic acid (EDTA), Elastatinal, lima bean trypsin inhibitor (LBTI), E-64, chymostatin and phenyl methyl sulfonyl fluoride (PMSF) in 50 mM phosphate buffer

(pH 7.5) for 30 min at 4°C and the residual enzyme activity was measured using Suc-Leu-Leu-Val-Tyr-MCA as a substrate (44). Control without inhibitor was taken as 100%.

Assay for HIV reverse transcriptase inhibitory activity The assay for HIV reverse transcriptase inhibitory activity was performed by using a nonradioactive ELISA kit from Boehringer-Mannheim (Germany) as described by Zhang et al. (5). The assay takes advantage of the ability of reverse transcriptase to synthesize DNA, starting from the template/primer hybrid poly(A) oligo(dT) 15. The digoxigenin and biotin-labeled nucleotides in an optimized ratio are incorporated into one of the same DNA molecules, which are freshly synthesized by the reverse transcriptase (RT). The detection and quantification of synthesized DNA as a parameter for RT activity allow sandwich ELISA protocol. Biotin-labeled DNA binds to the surface of microtiter plate modules that have been precoated with streptavidin. In the next step, an antibody to digoxigenin, conjugated to peroxidase, binds to the digoxigenin-labeled DNA. In the final step, the peroxidase substrate is added. The peroxidase enzyme catalyzes the cleavage of the substrates, producing a colored reaction product. The absorbance of the samples at 405 nm can be determined using a microtiter plate (ELISA) reader and is directly correlated to the level of RT activity. A fixed amount (4–6 ng) of recombinant HIV-1 reverse transcriptase was used. The inhibitory activity of the cordysobin was calculated as percent inhibition as compared to a control without the protein.

RESULTS

Enzyme purification The cordysobin activity of *C. sobolifera* was detected in the unadsorbed fraction D1 from the DEAE-cellulose column (Table 1). Subsequently, fraction D1 was subjected to an SP-Sepharose column and resolved into three fractions: SP1, SP2, and SP3. Protease activity resided in fraction SP3 (Table 1). Fraction SP3 was fractionated into a large active fraction, SU1, and a small inactive fraction, SU2, after gel filtration over a Superdex 75 column (Fig. 1, Table 1).

Determination of molecular mass and amino acid sequence The cordysobin appeared as a single band with an apparent molecular mass of 31 kDa in SDS-PAGE (Fig. 2A) and a single peak with the same molecular mass in gel filtration (Fig. 1), leading to the conclusion that the enzyme was a monomeric protein with a molecular mass of 31 kDa. The enzyme was purified 2910.35-fold from crude extract with a 7.37% yield and an activity of 56,780.98 U/mg of the purified protease (Table 1). The N-terminal sequence of the purified protease was AFSTQPGAVCGK, which showed no similarity to protein sequences of basidiomycete mushroom proteases or proteases from the ascomycete filamentous fungi *H. lacunosa*, *Penicillium chrysogenum*, and *Paecilomyces lilacinus*. However, it demonstrated low homology to proteases from the ascomycete mushrooms *C. sinensis*, *C. chlamydosporia*, *C. militaris*, *Aspergillus clavatus*, *A. fumigatus*, and *A. oryzae* (Table 2). The peptides from a tryptic digest were analyzed by ESI-MS/MS, which revealed two major peaks at m/z 372.5 and 1196.8 (data not shown), identified as ASFSNYGR and SMVLNKASIPVPLKECK. Database searches using BLAST indicated that the two amino acid sequences showed 100% homology to *Rhodothermus marinus* thermitase and *Tribolium castaneum* serine protease, respectively.

Substrate specificities toward various MCA-substrates The hydrolysis of various MCA-substrates by cordysobin was shown in Table 3. The purified protease hydrolyzed Suc-Leu-Leu-Val-Tyr-MCA most efficiently among the substrates used. The cordysobin revealed low activity against Boc-Phe-Ser-Arg-MCA, a substrate for trypsin. No cleavage to Suc-Ala-Pro-Ala-MCA, Z-Phe-Arg-MCA, Z-Arg-Arg-MCA and Arg-MCA, which are substrates for elastase, cathepsin L, cathepsin

TABLE 1. Yields and protease activities of *Cordyceps sobolifera* chromatographic fractions at the various stages of purification (from 25 g dried fruiting body).

Fraction	Yield (mg)	Specific activity (units/mg)	Total activity (units)	Recovery of activity (%)	Fold purification
Crude extract	15012.56	19.51	2.93×10^5	100	1
$(\text{NH}_4)_2\text{SO}_4$ precipitation	1965.20	101.7	1.99×10^5	67.92	5.21
D1	33.03	1949.83	6.44×10^4	21.98	99.94
SP3	0.86	36395.35	3.13×10^4	10.57	1865.47
SU1	0.38	56780.98	2.16×10^4	7.37	2910.35

Enzyme assay conditions: 37°C/15 min, in 0.1 M Tris-HCl, pH 7.5.

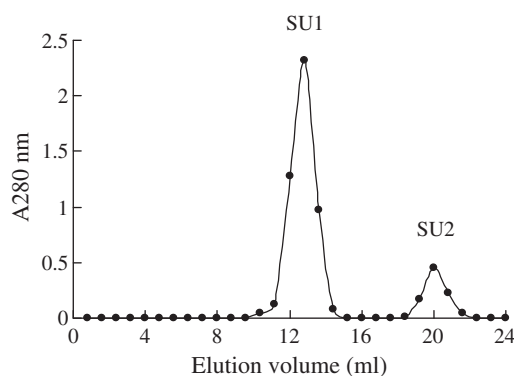


FIG. 1. Gel filtration of SP3 on a Superdex 75 HR10/30 column. A Superdex 75 (HR 10/30) column was eluted with 0.15 M NH_4HCO_3 buffer (pH 8.5). The flow rate was 0.4 ml/min. Fraction SU1 represents the purified protease.

B and aminopeptidase, respectively, was identified, suggesting that cordysobin is chymotrypsin-type.

Physiochemical properties of enzyme The pH dependence of cordysobin was shown in Fig. 2B. The activity against Suc-Leu-Leu-Val-Tyr-MCA was measured on a pH range of 4.0–12.0. Optimum was found at pH 10.0. The optimal temperature was found to be approximately 65°C (Fig. 2C). About 20% decrease in activity was observed at 70°C. The enzyme was thermally stable at temperatures lower than 40°C. Its activity was slightly decreased at 50°C and was not detectable at temperatures exceeding 60°C (Fig. 2D). The K_m of cordysobin against Suc-Leu-Leu-Val-Tyr-MCA was 0.41 μM at pH 10.0 and 37°C. The V_{max} was 13.44 $\mu\text{M} \cdot \text{min}^{-1}$.

In the effects of various cations on the activity of cordysobin assays, all of the ion cations except Fe^{2+} examined exerted a dose-dependent inhibitory action on protease activity at the concentration

TABLE 2. N-terminal sequence of *C. sobolifera* protease compared to other fungal alkaline serine proteases (results of a BLAST search).

Species	Phylum	N-terminal sequence	Reference
<i>Cordyceps sobolifera</i>	Ascomycota	AFSTQP GA VCGK	This study
<i>Cordyceps chlamydosporia</i>	Ascomycota	<u>A</u> IVE <u>Q</u> QGA P W	(Accession number: CAD20579)
<i>Cordyceps sinensis</i>	Ascomycota	ALATQ HGAPW	49
<i>Cordyceps militaris</i>	Ascomycota	YQXXVTFXDF/ VSXXGDSGVGGN/ NAFNDYTFK	45
<i>Aspergillus clavatus</i>	Ascomycota	APTHQ EGAPWGLAAI	47
<i>Aspergillus clavatus EST1</i>	Ascomycota	<u>AL</u> T <u>Q</u> S G APWGLGSI	44
<i>Aspergillus fumigatus</i>	Ascomycota	<u>AL</u> T <u>Q</u> K G APWGLGSI	48
<i>Aspergillus oryzae</i>	Ascomycota	TQT NAPWGLA	58
<i>Agaricus bisporus</i>	Basidiomycota	MHFSLSFATL	22
<i>Helvella lacunosa</i>	Ascomycota	ANVVQWPVPC	4
<i>Paecilomyces lilacinus</i>	Ascomycota	ARAPLLTPRG	59
<i>Penicillium chrysogenum</i>	Ascomycota	MGFLKLLSTS	60
<i>Pleurotus citrinopileatus</i>	Basidiomycota	VCQCNPAPWGL	30
<i>Pleurotus eryngii</i>	Basidiomycota	GPQFPEA	28

Identical amino acid residues are in bold and underscored.

range of 1.25–10 mM. Hg^{2+} showed strong inhibitory activity, with a remaining protease activity of 12.19% at a concentration of 1.25 mM. In contrast, Fe^{2+} had a stimulatory effect on protease activity at the low concentration range of 1.25–10 mM (data not shown).

The effects of various protease inhibitors on cordysobin are summarized in Table 4. Activity was strongly inhibited by chymostatin and serine protease inhibitor PMSF. Furthermore, different concentrations (0.6 mM, 0.1 mM, 0.05 mM, and 0.01 mM)

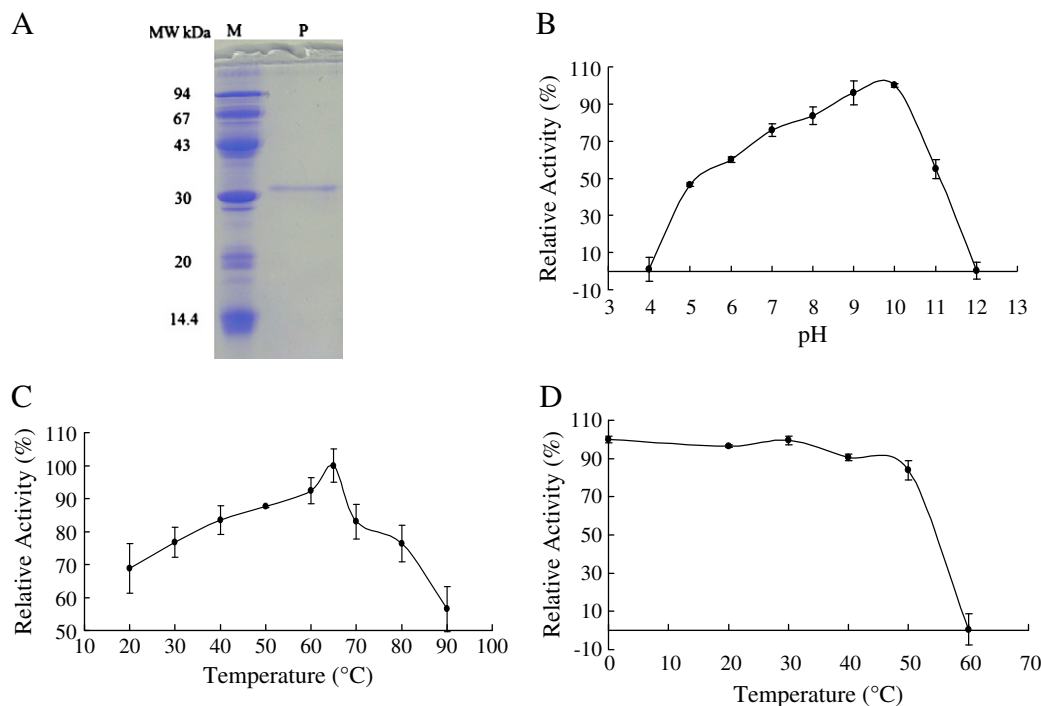


FIG. 2. Characterization of the purified protease from *Cordyceps sobolifera*. (A) SDS-PAGE of SU1 (*C. sobolifera* protease designated as cordysobin). The apparent molecular mass of SU1 was 31 kDa. (B) Effect of pH on protease activity of cordysobin. The activity at pH 10.0 was taken as 100%. The results shown are mean \pm SD ($n = 3$). The SD of each data point was less than 7%. (C) Effect of temperature on protease activity of cordysobin. The activity at 65°C was taken as 100%. The results shown are mean \pm SD ($n = 3$). The SD of each data point was less than 8%. (D) Thermal stability of the purified protease. The enzyme was incubated at different temperatures for 40 min, followed by measurement of the residual protease activity using the standard assay. The protease activity tested at 0°C was regarded as 100%. The results shown are mean \pm SD ($n = 3$). The SD of each data point was less than 8%.

TABLE 3. Substrate specificity of the serine protease toward methyl-coumaryl-7-amide (MCA) substrates.

Substrates (1 mM)	Relative activity (%)
Suc-Leu-Leu-Val-Tyr-MCA	100.00 ± 3.41 ^a
Boc-Phe-Ser-Arg-MCA	1.49 ± 2.27 ^b
Suc-Ala-Pro-Ala-MCA	0 ^c
Z-Phe-Arg-MCA	0 ^c
Z-Arg-Arg-MCA	0 ^c
Arg-MCA	0 ^c

The proteolytic activity assays were performed in duplicate at pH 8.0. Different superscripts (e.g., a, b, c) indicate a statistically significant difference ($p < 0.05$). MCA, methyl-coumaryl-7-amide; Boc, butyloxycarbonyl.

of chymostatin and PMSF toward cordysobin have been carried out. The remaining protease activity were 0.54%, 0.60%, 0.81%, and 5.23% for chymostatin, and 0.61%, 11.26%, 36.73%, and 64.34% for PMSF. On the other hand, the aspartic protease inhibitor Pepstatin A, the metalloprotease inhibitor EDTA, and the elastase protease inhibitor Elastatinal scarcely showed any inhibitory effect. Trypsin protease inhibitor LBTI and cysteine protease inhibitor E-64 slightly inhibited the activity. Combining the sensitivity to inhibitors with its mass spectrometry result and substrate specificity, it could be concluded that the cordysobin is a chymotrypsin-type serine protease.

The HIV-1 RT inhibitory activity of cordysobin at concentrations of 5.2×10^{-3} μ M, 2.6×10^{-2} μ M, and 1.3×10^{-1} μ M was 32.07%, 96.20%, and 100%, respectively. The IC_{50} value was 8.2×10^{-3} μ M.

DISCUSSION

In the present study, cordysobin, a novel serine protease from fruiting bodies of the fungus *C. sobolifera*, was purified to homogeneity by a three-step purification scheme, and its molecular properties were characterized. Cordysobin appeared as a single band on SDS-PAGE stained by Coomassie Brilliant Blue R-250, confirming that the purification procedure was able to isolate cordysobin from all other proteins. The results of electrophoretic analyses and gel-filtration chromatography indicate that cordysobin is a 31 kDa monomer. The molecular mass of cordysobin (31 kDa) is close to serine proteases from *H. lacunosa* (33.5 kDa), *A. clavatus* EST1 (32 kDa), *A. clavatus* (35 kDa), *A. fumigatus* (32 kDa), and *C. sinensis* (31 kDa) (4,44,48–50), but larger than those of proteases from *A. mellea* (23–24), *G. frondosa* (26), *P. eryngii* (28), *P. ostreatus* (29), *P. citrinopileatus* (30), and *T. saponaceum* (31). However, its N-terminal sequence shows only slight similarity to fungal serine proteases from *C. sinensis* (50), *A. fumigatus* (49), *A. clavatus* EST1 (44), and *A. clavatus* (48) (Table 2). These results suggest that cordysobin might be a novel

protein and the present report of a protease cordysobin from *C. sobolifera* thus adds to the literature.

Proteases have been purified from a few mushroom species, including serine proteases from the genus *Coprinus* (51) and *A. bisporus* (52); an aminopeptidase (53) and a prolyl endopeptidase (27) from *L. cinerascens*; a prolyl endopeptidase from *A. bisporus* (54); metalloproteases from *A. mellea* (23), *G. frondosa* (26), and *T. saponaceum* (31); a subtilisin-like protease from *P. ostreatus* (29); and a trypsin-like protease from *C. militaris* (45). In this study, cordysobin is strongly inhibited by chymotrypsin and PMSF. Combined with its 100% homology to serine protease in mass spectrometry result and the substrate specificity result that it specially cleaved the chymotrypsin substrate Suc-Leu-Leu-Val-Tyr-MCA but did not reveal any activity to other MCA-substrates (Table 3), the present result provided strong evidence that cordysobin was a chymotrypsin-type serine protease.

Under normal circumstances, at a certain temperature range as the temperature rises, enzyme activity gradually increased. In our study, the optimum temperature of cordysobin is 65°C. However, the thermal stability of cordysobin indicates there is no proteolytic activity at 60°C for 40 min, while the proteolytic activity at 50°C for 40 min is slightly decreased. It suggested that the cordysobin achieved its highest activity at 65°C for 15 min, but at this temperature it was unstable for a long time, and would lose its activity incubating for 40 min. An alkaline serine-protease from *A. clavatus* ES1 was reported to manifest an optimal temperature not matching its thermostability, which was just the same as that of cordysobin (44). The fact that cordysobin displayed a broad alkaline pH optimum (7.0 to 10.0) indicates that it is an alkaline protease. Owing to its high catalytic efficiency at alkaline condition, the cordysobin secreted by *C. sobolifera* was more valuable than the generally used proteases in some special industries such as leather softening and environmental bioremediation.

Some laccases (55), lectins (7), ribonucleases (5), peptides (56), and ribosome inactivating proteins (57) from mushrooms have been reported to inhibit HIV-1 RT. A comparison of cordysobin and other mushroom proteins in inhibiting HIV-1 RT indicates that cordysobin, the highest HIV-1 RT inhibitory activity with an IC_{50} value of 8.2×10^{-3} μ M, is the first protease reported in Ascomycota. It suggests that cordysobin might have potential applications for AIDS treatments.

In summary, an alkaline serine protease, cordysobin, with a distinctive N-terminal sequence and extremely high HIV-1 RT inhibitory activity was purified from the medicinal mushroom *C. sobolifera*. It manifested an optimal pH of 10.0, an optimum temperature of 65°C, a K_m of 0.41 μ M, a V_{max} of $13.44 \mu\text{M} \cdot \text{min}^{-1}$, and an IC_{50} value of 8.2×10^{-3} μ M toward HIV-1 RT. This protease might be helpful for screening AIDS therapeutic agents.

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TABLE 4. Effect of enzyme inhibitors on the activity of *C. sobolifera* serine protease.

Inhibitors	Protease activity remaining (%)
Pepstatin A (0.2 mM)	103.27 ± 4.33 ^a
EDTA (1 mM)	102.16 ± 1.27 ^a
Elastatinal (1 mM)	99.78 ± 3.84 ^a
LBTI (0.25 mM)	94.31 ± 4.09 ^b
E-64 (1 mM)	93.04 ± 1.47 ^b
Chymostatin (1 mM)	0.42 ± 0.21 ^c
PMSF (1 mM)	0.19 ± 1.53 ^c

The results shown are mean ± 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.

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