J. Microbiol. Biotechnol. (2011), **21**(3), 256–262
doi: 10.4014/jmb.1010.10060
First published online 26 January 2011

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

A Novel Metalloprotease from the Wild Basidiomycete Mushroom *Lepista nuda*

Wu, Y. Y.[1], H. X. Wang[1]*, and T. B. Ng[2]*

[1]*State Key Laboratory for Agrobiotechnology and Department of Microbiology, China Agricultural University, Beijing 100193, China*
[2]*School of Biomedical Sciences, Faculty of Medicine, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong, China*

Received: October 26, 2010 / Revised: December 17, 2010 / Accepted: December 18, 2010

A 20.9-kDa metalloprotease was isolated from dried fruiting bodies of the wild basidiomycete mushroom *Lepista nuda*. The N-terminal amino acid sequence of the protease was seen to be ATFVLTAATNTLFTA, thus displaying no similarity with the sequences of previously reported metalloproteases. The protease was purified using a procedure that entailed ion-exchange chromatography on CM-Cellulose, Q-Sepharose, and Mono S, and FPLC-gel filtration on Superdex 75. The protease functioned at an optimum pH of 7.0 and an optimum temperature of 50°C. It was also noted that the protease demonstrated a proteolytic activity of 1,756 U/mg toward casein. The K_m of the purified protease toward casein was 6.36 mg/ml at a pH of 7.0 and with a temperature of 37°C, whereas the V_{max} was 9.11 μg ml^{-1} min^{-1}. The activity of the protease was adversely affected by EDTA-2Na, suggesting that it is a metalloprotease. PMSF, EGTA, aprotinin, and leupeptin exerted no striking inhibitory effect. The activity of the protease was enhanced by Fe^{2+}, but was curtailed by Cd^{2+}, Cu^{2+}, Hg^{2+}, Pb^{2+}, Zn^{2+}, and Fe^{3+} ions. The protease also exhibited inhibitory activity against HIV-1 reverse transcriptase with an IC_{50} value of 4.00 μM. The IC_{50} values toward hepatoma Hep G2 and leukemia L1210 cells *in vitro* were 4.99 μM and 3.67 μM, respectively.

Keywords: Mushroom, metalloprotease, *Lepista nuda*, purification

Mushrooms produce a large number of proteins comprising proteases [12, 41], antifungal proteins [29], lectins [6, 30], ribosome inactivating proteins [31, 33], polysaccharides [37, 42], and polysaccharide–peptides and polysaccharide–protein complexes [34, 35]. Some of these mushroom proteins may have potentially curative or health-promoting activities for human beings [17, 21, 28]. It is known that proteases hydrolyze proteins with substrate specificity. They can also be produced in bulk quantities using microbial approaches [1, 14]. Proteases find diverse applications in the brewing, dairy, meat, detergent, leather, and photographic industries [10]. Worldwide sales of industrial enzymes amount to billions of dollars annually. A substantial percentage of these enzyme sales are in the form of detergents [8].

Metalloprotease is a kind of protease of which the activity is reduced by EDTA-2Na. There are several metalloproteases purified from mushrooms, including *Armillariella mellea* [12], *Pleurotus ostreatus* [26], *Tricholoma saponaceum* [13], *Flammulina velutipes*, *Lentinus edodes*, *Ganoderma lucidum*, and *Grifola frondosa* [22]. In a preliminary experiment, we found that the extract of *Lepista nuda* fruiting bodies showed strong protease activity owing to the presence of a metalloprotease. Hence, in this study, we describe the purification and characterization of a new metalloprotease from the dried fruiting bodies of *Lepista nuda*.

The literature on *Lepista nuda* and the *Lepista* species in general is not abundant. The accumulation of heavy metals by *Lepista nuda* has been reported [7, 9, 20]. *Lepista nuda* have been found to contain very useful chemical components, and possess interesting biological properties [2]. The trace metal contents of *Lepista nuda* have been previously determined [24]. *Lepista nuda* is known to exhibit laccase activity [27] and antimicrobial activity [5]. Finally, some isolates have been obtained from *Lepista nuda*, including ceramide constituents [38], and sterols and triterpenoids [39].

Materials and Methods

Materials

Dried fruiting bodies of the mushroom *Lepista nuda* were purchased in the Heilongjiang Province in China. The Q-Sepharose, Mono S, Superdex 75 10/300 GL column, and AKTA Purifier were all obtained from GE Healthcare (USA). CM-Cellulose and casein sodium salt

**Corresponding author*
H.X. Wang
Phone: +86-10-62732578; Fax: +86-10-62732578;
E-mail: hxwang@cau.edu.cn
T.B. Ng
Phone: +852-2609-8031; Fax: +852-2609-8031;
E-mail: b021770@mailserv.cuhk.edu.hk

were acquired from Sigma-Aldrich (USA). All other chemicals used were of reagent grade.

Isolation of the Protease

Dried fruiting bodies of the mushroom (10 g) were homogenized in 0.15 M NaCl at 4°C. The homogenate was stirred overnight at 4°C before centrifugation at 8,000 rpm for 15 min. The precipitate was then dissolved and dialyzed to remove NaCl before being applied to a column (10×100 cm) of CM-Cellulose, which had previously been equilibrated, and was then eluted with a 10 mM NaAc-HAc buffer (pH 4.0). After removal of the unadsorbed peak (C1) containing no protease activity, four adsorbed peaks, C2, C3, C4, and C5, were eluted with 50 mM NaCl, 150 mM NaCl, 300 mM NaCl, and 1 M NaCl in the starting buffer, respectively. Protease activities toward casein in the chromatographic fractions were monitored as described below. The active fraction (C3) was applied to a column (1.5×30 cm) of Q-Sepharose, which had been equilibrated, and then eluted with a 10 mM Tris-HCl buffer (pH 7.2). Unbound material was eluted with the starting buffer, whereas bound material was desorbed by the addition of 50 mM NaCl, 150 mM NaCl, and 1 M NaCl in the starting buffer. The active peak (Q2) was then subjected to a Mono S column (4.6/100 PE), which had been equilibrated, and was then eluted with a phosphate buffer (pH 6.0). After removal of unbound material, the adsorbed material was fractionated using a linear concentration gradient of 0−1 M NaCl in a 10 mM phosphate buffer (pH 6.0). The active fraction (S3) was subsequently chromatographed on a Superdex 75 10/300 GL column (GE Healthcare, USA) in a 0.15 M NH_4HCO_3 buffer (pH 8.5) using an AKTA Purifier (GE Healthcare, USA). Only one peak (SU1) was obtained, which represented purified protease.

Determination of Molecular Mass and the N-Terminal Sequence

The active peak (SU1) was subsequently analyzed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) [15]. The molecular mass of the purified protein was also determined in SDS-PAGE, as well as by FPLC-gel filtration as described above. N-Terminal sequencing of the protein was carried out using an HP G-1000A Edman degradation unit and an HP 1000 HPLC system [16].

Assay for Protease Activity

A solution of casein, which was used as a substrate in the protease assay, was freshly prepared as follows. To 0.1 g of casein, 10 ml of 200 mM phosphate buffer (pH 7.5) was added. Subsequently, the solution was heated to 60°C for 30 min. The precipitate was removed and the resulting solution utilized [11]. The test sample, or trypsin solution (20 µl), was mixed with 180 µl of the above casein solution and the reaction mixture was incubated at 37°C for 15 min. Following on from this, 200 µl of 5% trichloroacetic acid was added. The reaction mixture was allowed to stand at room temperature for 30 min before centrifugation at 12,000 rpm for 5 min. The absorbance of the supernatant was read at 280 nm against water as the blank. One unit of protease activity was defined as an absorbance increase of 0.001 at 280 nm per milliliter of reaction mixture per minute under the experimental conditions.

Determination of the Optimum pH and Temperature of the Protease

In the assay for the determination of the optimum pH and temperature, a solution of casein, which was used as substrate, was freshly prepared as described previously [32]. The assay buffers were prepared in a 200 mM citrate buffer (pH 2.0−8.0), or a Tris-HCl buffer (pH 8.0−9.0). The purified protease (20 µl) was incubated at 37°C for 15 min with 80 µl of 1% casein solution (pH 7.5) and 100 µl of assay buffer, as described above. The reaction was subsequently ended by the addition of 200 µl of 5% trichloroacetic acid. The reaction mixture was allowed to stand at room temperature for 30 min before centrifugation at 12,000 rpm for 5 min. The absorbance of the supernatant was read at 280 nm against water as the blank. To determine the optimum temperature, the reaction mixture was incubated at between 4°C and 90°C for 15 min. The assay buffer was a 200 mM phosphate buffer (pH 7.5).

Enzyme Kinetics

In the study of enzyme kinetics, the protease solution (20 µl) was incubated at the optimum pH and at 37°C for 15 min with 180 µl of 0.625 mg/ml, 1.25 mg/ml, 2.5 mg/ml, 5 mg/ml, or 10 mg/ml casein solution. The reaction was terminated by the addition of 200 µl of 5% trichloroacetic acid. The reaction mixture was then allowed to stand at room temperature for 30 min before centrifugation at 12,000 rpm for 5 min. The absorbance of the supernatant was read at 280 nm against water as the blank [18].

Assay of Mechanistic Class

In the assay for the determination of the mechanistic class that the protease belongs to, the protease was exposed to the following inhibitors: PMSF, EGTA, aprotinin, EDTA-2Na, and leupeptin (Amresco). Residual enzyme activity was measured by the method described above.

Assay of Effects of Metal Ions

The enzyme solution (10 µl) was preincubated at 37°C for 30 min at a pH of 7.5 with 10 µl of different metal ions and chemical reagents at concentrations ranging from 1.25 mM to 10 mM [19]. Protease activity was then assayed by the methods previously described.

Assay for Antiproliferative Activity on Tumor Cell Lines

The antiproliferative activity of the purified protease was determined as follows. The cell lines Hep G2 and L1210 were maintained in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 mg/l streptomycin, and 100 IU/ml penicillin, at 37°C in a humidified atmosphere of 5% CO_2. Cells (1×10^4), in their exponential growth phase, were seeded into each well of a 96-well culture plate and incubated for 3 h, before the addition of the purified protease, and then incubated for another 48 h. A radioactive precursor, 1 µCi [methyl-^{3}H] thymidine (GE Healthcare, USA), was then added to each well and incubation continued for a further 6 h before the cultures were harvested by means of a cell harvester. The incorporated radioactivity was determined by liquid scintillation counting [36].

Assay for HIV-1 Reverse Transcriptase (RT) Inhibitory Activity

The assay for HIV-1 RT inhibitory activity was assessed by using an enzyme-linked immunosorbent assay (ELISA) kit acquired from Boehringer Mannheim (Germany). The assay took advantage of the ability of RT to synthesize DNA, starting from the template/primer hybrid poly(A) oligo$(dT)_{15}$. The digoxigenin- and biotin-labeled nucleotides, in an optimized ratio, were incorporated into one of the same DNA molecules, which had been freshly synthesized by the

Table 1. Yields and protease activities (toward casein) of various chromatographic fractions (from 10 g dried *Lepista nuda* fruiting bodies).

Chromatographic fraction	Yield (mg)	Specific protease activity (U/mg)	Total protease activity (U)	Purification fold
Crude extract	1,157.0	114	131,898	1
C1	66.7	<4	<267	-
C2	175.4	<4	<717	-
C3	**119.0**	**402**	**47,838**	**4**
C4	196.3	<4	<785	-
C5	224.5	24	<5,388	-
Q1	15.4	<4	<62	-
Q2	**13.7**	**604**	**8,275**	**5**
Q3	26.3	30	789	-
Q4	48.1	<4	<192	-
S1	3.3	<4	<13	-
S2	4.6	<4	<18	-
S3	**3.5**	**1,524**	**5,334**	**13**
SU1	**2.7**	**1,756**	**4,741**	**15**

RT. The detection and quantification of synthesized DNA as a parameter for RT activity followed sandwich ELISA protocols. Biotin-labeled DNA was bound to the surface of microtiter plate modules that had been precoated with streptavidin. An antibody to digoxigenin, conjugated to peroxidase (anti-DIG-POD), was subsequently bound to the digoxigenin-labeled DNA. Finally, the peroxidase substrate was added. The peroxidase enzymes catalyzed the cleavage of the substrate and produced a colored reaction product. The absorbance of the samples at 405 nm could be determined by using a microtiter plate (ELISA) reader and was directly correlated to the level of RT activity. A fixed amount (4–6 ng) of recombinant HIV-1 RT was used. The inhibitory activity of the protease was calculated as the percentage of inhibition as compared with a control without the protein [40].

RESULTS

Ion-exchange chromatography of *Lepista nuda* extract on CM-Cellulose produced an adsorbed fraction, C3, with the highest protease activity. Little or no protease activity was observed in the other fractions. Subsequently, fraction C3 was resolved into an unadsorbed fraction Q1 and adsorbed fractions Q2, Q3, and Q4. Fraction Q2 was seen to have a much higher protease activity than the other fractions (Table 1). Ion-exchange chromatography of fraction Q2 on Mono S resulted in a small unadsorbed fraction, S1, and two adsorbed fractions, S2 and S3, with the protease activity residing in the latter fraction S3 (Fig. 1, Table 1). Fraction S3 appeared as a single active peak, SU1, upon FPLC-gel filtration on Superdex 75 (Fig. 2, Table 1). The protease appeared as a single band with a molecular mass of 20.9 kDa in SDS–PAGE (Fig. 3) and in gel filtration (Fig. 2). The increase of protease activity toward casein, throughout the various stages of purification, is shown in Table 1. An approximately 15-fold purification was obtained. The N-terminal amino acid sequence of the protease was noted as being ATFVLTAATNTLFTA, which showed no similarity with the protein sequences of the metalloproteases previously reported in the literature (Table 2).

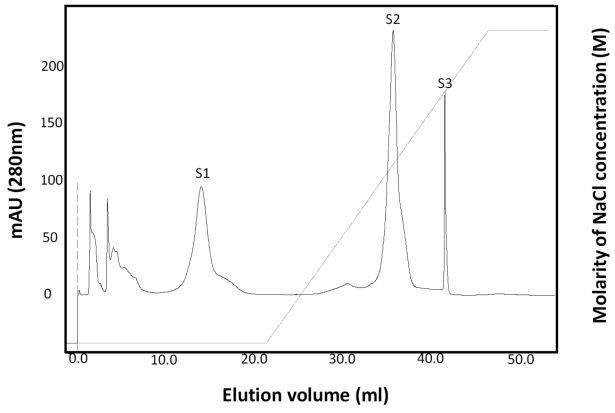

Fig. 1. Anion-exchange chromatography of fraction Q2, from a Q-Sepharose column, on a Mono S column (4.6/100 PE).
The column was first equilibrated and eluted with a 10 mM phosphate buffer (pH 6.0) and then with a linear gradient of 0–1 M NaCl in the same buffer (shown as an oblique line).

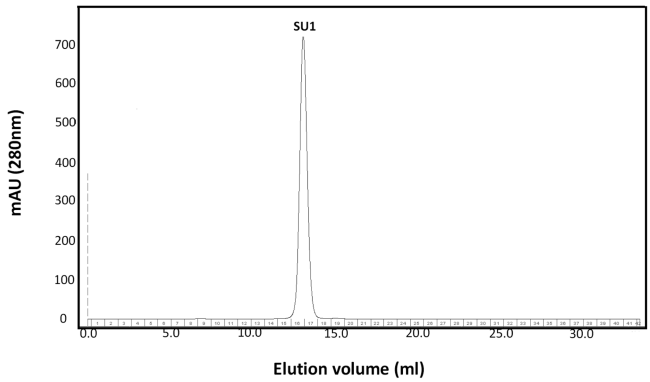

Fig. 2. Gel filtration of S3 on a Superdex 75 10/300 GL column.
The molecular mass of SU1 was 20.9 kDa.

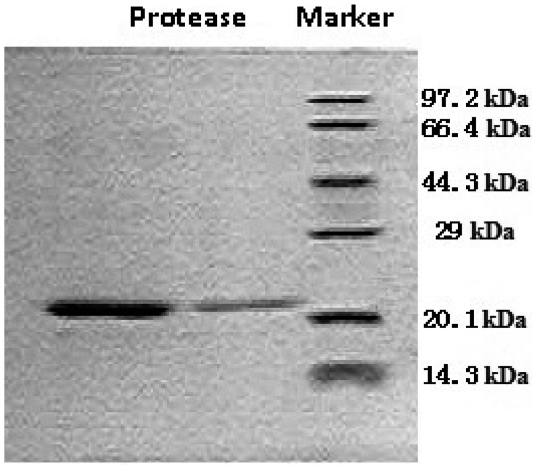

Fig. 3. SDS–PAGE of SU1 representing the *Lepista nuda* protease.
The molecular mass of SU1 was 20.9 kDa. The two bands represent proteases at different concentrations.

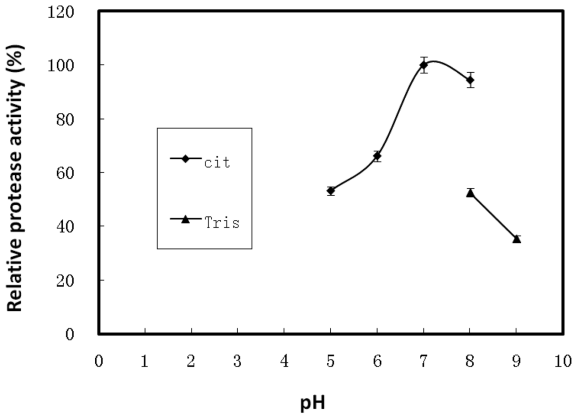

Fig. 4. Effect of pH on the activity of *Lepista nuda* protease.
Cit: citrate buffer; Tris: Tris-HCl buffer. Results represent the mean ± SD of triplicate determinations (n=3).

The K_m of the purified protease for casein was 6.36 mg/ml at a pH of 7.0 and temperature of 37°C. The V_{max} was 9.11 μg ml^{-1} min^{-1}. The protease exhibited an optimum pH of 7.0 (Fig. 4) and an optimum temperature of 50°C (Fig. 5). There was minimal activity at a pH of 9.0. The activity of the protease was adversely affected by EDTA-2Na, but not affected to any major extent by PMSF, EGTA, aprotinin, and leupeptin (Table 3), indicating that it is a metalloprotease. The activity of the protease was potentiated by Fe^{2+}, but reduced in the presence of Cd^{2+}, Cu^{2+}, Hg^{2+}, Pb^{2+}, Zn^{2+}, and Fe^{3+} ions (Table 4). The protease also exhibited an inhibitory activity against HIV-1 RT with an IC_{50} of 4.00 μM. The IC_{50} values toward the Hep G2 and L1210 cells *in vitro* were 4.99 μM and 3.67 μM, respectively (Table 5).

Discussion

The isolation of proteases from some mushroom species has previously been reported. These include serine proteases from the *Coprinus* species [25], the *Agaricus bisporus* species [4], an aspartic protease from the *Pleurotus eryngii* species [33], and a subtilisin-like protease from *Pleurotus ostreatus* [23]. Metalloproteases have also been purified from *Armillariella mellea* [12], *Tricholoma saponaceumi* [13], *etc*.

The characteristics of the *Lepista nuda* metalloprotease were seen to resemble those of other mushroom metalloproteases, but there were also a number of remarkable differences (Table 6). The *Lepista nuda* metalloprotease remained unadsorbed on DEAE-Cellulose, whereas many other metalloproteases were adsorbed and could be purified by anion-exchange chromatography on DEAE-Cellulose [12, 13, 26]. The molecular mass of the *Lepista nuda* metalloprotease was noted as 20.9 kDa, which is similar to most other metalloproteases, but much lower than that of the metalloprotease from *Pleurotus ostreatus* (32 kDa) [26]. The *Lepista nuda* metalloprotease was most active at a pH of 7.0, suggesting it is a neutral protease, whereas the *Grifola frondosa* metalloprotease is an alkaline protease

Table 2. N-Terminal sequence of the isolated *Lepista nuda* metalloprotease as compared with other mushroom metalloproteases (results of BLAST search).

Species	N-Terminal sequence
Lepista nuda	ATFVLTAATNTLFTA
Armillariella mellea	XXYNGXTXSRQTTLV
Tricholoma saponaceum	ALYVGXSPXQQSLLV
Grifola frondosa	TYNGCSSSEQSALAA
Coprinopsis cinerea	MRLSSLLTSLVYATALV
Laccaria bicolor	MSPFTAKWLTYRKIGP
Nidula niveotomentosa	RPPGQVPLHLGNRIES
Pleurotus ostreatus	MLRSILLIALSCSAYVL

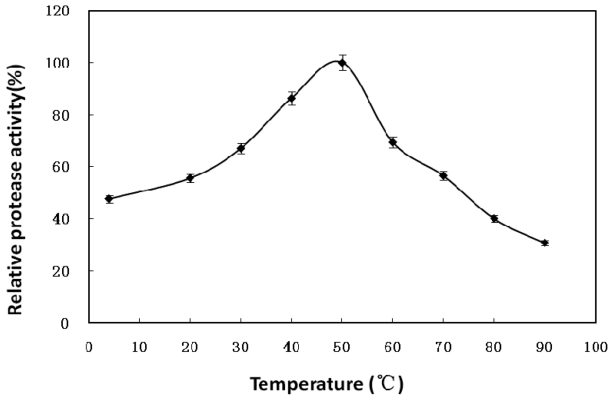

Fig. 5. Effect of temperature on the activity of *Lepista nuda* protease.
Results represent the mean ± SD of triplicate determinations (n=3).

Table 3. Effects of protease inhibitors on the activity of *Lepista nuda* protease.

Inhibitors	Remaining protease activity (%)	Inhibitors	Remaining protease activity (%)
PMSF		EGTA	
0.04 mM	102.8±3.9	0.04 mM	100.4±0.9
0.2 mM	97.4±3.0	0.2 mM	98.0±3.9
1 mM	94.7±2.1	1 mM	87.2±3.0
Aprotinin		Leupeptin	
0.04 mM	116.2±1.1	0.04 mM	100.3±3.9
0.2 mM	104.6±1.0	0.2 mM	99.4±2.2
1 mM	102.4±3.7	1 mM	98.3±3.2
EDTA-2Na			
0.04 mM	95.4±3.6		
0.2 mM	61.2±0.8		
1 mM	16.9±1.5		

Results represent the mean ± SD (n=3).

Table 4. Effects of metal ions and chemical reagents on the activity of *Lepista nuda* protease.

Metal ion concentration	Relative protease activity (%)			
	10 mM	5 mM	2.5 mM	1.25 mM
Fe^{2+}	95	165	156	138
K^{+}	101	98	104	103
Ca^{2+}	91	92	96	99
Cd^{2+}	17	51	64	73
Cu^{2+}	32	53	66	72
Hg^{2+}	10	29	51	65
Mg^{2+}	93	93	98	101
Mn^{2+}	85	95	98	99
Pb^{2+}	40	84	91	97
Zn^{2+}	45	91	101	105
Al^{3+}	84	109	107	109
Fe^{3+}	21	82	121	99

Protease was preincubated at 37°C for 15 min at pH 7.0 with a phosphate buffer used as the blank (activity taken as 100).

because its optimum functional pH is in the range of 9.0–10.0 [22]. The optimum functional temperature of the *Lepista nuda* metalloprotease is 50°C, in accord with the metalloproteases from *Armillariella mellea* and *Tricholoma saponaceum*, but higher than the *Pleurotus ostreatus* metalloprotease (35°C) [26]. The thermostability and the metal ions effects vary from one mushroom metalloprotease to another. For example, Fe^{2+} ions were seen to enhance the protease activity of the *Lepista nuda* metalloprotease but reduced that of the *Pleurotus ostreatus* metalloprotease. However, Hg^{2+} reduced the activity of the majority of mushroom metalloproteases [12, 13, 22]. The N-terminal sequence of the *Lepista nuda* metalloprotease was dissimilar from proteases previously reported.

The isolated protease was characterized by its ability to inhibit the proliferation of two tumor cell lines, Hep G2 and L1210. Antiproliferative activity has been detected in most mushroom lectins, but has rarely been observed in mushroom proteases. Thus, the potent antiproliferative activity of the *Lepista nuda* protease makes it a promising candidate for cancer therapy. HIV-1 RT is a key enzyme of the HIV life cycle. Screening of HIV-1 RT inhibitors is currently a strategy in the ongoing search for anti-HIV drugs. The *Lepista nuda* protease demonstrated HIV-1 RT inhibitory activity, which has also been detected in the *Pleurotus eryngii* protease [32]. It is likely that the mechanism of inhibition is analogous to the protein–protein interaction involved in the inhibition of HIV-1 RT

Table 5. Inhibition rate (%) of the *Lepista nuda* protease on Hep G2 cells, L1210 cells, and HIV-1 RT.

	Inhibition (%)				IC_{50} (μM)
	8 μM	4 μM	2 μM	1 μM	
HIV-RT	72.3±7.0	59.8±5.2	41.0±4.4	20.1±2.9	4.00
Hep G2	67.6±6.5	51.3±6.0	32.4±4.1	12.9±1.4	4.99
L1210	75.5±6.8	60.7±5.4	43.6±4.3	22.3±2.8	3.67

Results are presented as the mean ± SD (n=3).

Table 6. Comparison of the characteristics of five mushroom metalloproteases.

Mushroom	*Lepista nuda*	*Armillariella mellea*	*Pleurotus ostreatus*	*Tricholoma saponaceum*	*Grifola frondosa*
Absorbed ion exchange	CM-Cellulose Q-Sepharose Mono S	DEAE-Cellulose Mono Q	CM-Cellulose DEAE-Cellulose	DEAE-Cellulose	CM-Cellulose
Molecular mass (kDa)	20.9	18.5	32	18	20
Optimum pH	7.0	7.0	6.5	7.5	9.0–10.0
Optimum temperature (°C)	50	55	35	55	ND
Temperature stability (°C)	⩽50	⩽60	ND	⩽30	⩽80
Reduced ions	Cd^{2+}, Cu^{2+}, Hg^{2+}	Hg^{2+}	Co^{2+}, Cu^{2+}, Fe^{2+}	Cu^{2+}, Hg^{2+}	Hg^{2+}
Enhanced ions	Fe^{2+}	UD	Ca^{2+}, Mg^{2+}, Zn^{2+}	UD	Mg^{2+}
References	This study	[12]	[26]	[13]	[22]

UD=undetectable; ND=not determined.

by the homologous protease [3]. Since *Lepista nuda* is an edible mushroom, its purified protease is a potential candidate for AIDS therapy.

In summary, the isolated mushroom protease of *Lepista nuda* is a metalloprotease with some very distinct and promising characteristics.

Acknowledgment

This work was financially supported through the National Grants of China (2010CB732202).

References

1. Adil, A. and S. Mohammed. 1998. Alkaline proteases: A review. *Bioresour. Technol.* **64:** 175–183.
2. Barros, L., B. A. Venturini, P. Baptista, L. M. Estevinho, and I. C. Ferreira. 2008. Chemical composition and biological properties of Portuguese wild mushrooms: A comprehensive study. *J. Agric. Food Chem.* **56:** 3856–3862.
3. Bottcher, M. and F. Grosse. 1997. HIV-1 protease inhibits its homologous reverse transcriptase by protein-protein interaction. *Nucleic Acids Res.* **25:** 1709–1714.
4. Burton, K. S., D. A. Wood, C. F. Thurston, and P. J. Barker. 1993. Purification and characterization of a serine proteinase from senescent sporophores of the commercial mushroom *Agaricus bisporus*. *J. Gen. Microbiol.* **139(Pt 6):** 1379–1386.
5. Dulger, B., C. C. Ergul, and F. Gucin. 2002. Antimicrobial activity of the macrofungus *Lepista nuda*. *Fitoterapia* **73:** 695–697.
6. Feng, K., Q. H. Liu, T. B. Ng, H. Z. Liu, J. Q. Li, G. Chen, H. Y. Sheng, Z. L. Xie, and H. X. Wang. 2006. Isolation and characterization of a novel lectin from the mushroom *Armillaria luteo-virens*. *Biochem. Biophys. Res. Commun.* **345:** 1573–1578.
7. Gabriel, J., P. Baldrian, K. Hladikova, and M. Hakova. 2001. Copper sorption by native and modified pellets of wood-rotting basidiomycetes. *Lett. Appl. Microbiol.* **32:** 194–198.
8. Hodgson, J. 1994. The changing bulk biocatalyst market. *Biotechnology* (*NY*) **12:** 789–790.
9. Kalac, P., J. Burda, and I. Staskova. 1991. Concentrations of lead, cadmium, mercury and copper in mushrooms in the vicinity of a lead smelter. *Sci. Total Environ.* **105:** 109–119.
10. Kalisz, H. M. 1988. Microbial proteinases. *Adv. Biochem. Eng. Biotechnol.* **36:** 1–65.
11. Keay, L. and B. S. Wildi. 1970. Proteases of the genus *Bacillus*. I. Neutral proteases. *Biotechnol. Bioeng.* **12:** 179–212.
12. Kim, J. H. and Y. S. Kim. 1999. A fibrinolytic metalloprotease from the fruiting bodies of an edible mushroom, *Armillariella mellea*. *Biosci. Biotechnol. Biochem.* **63:** 2130–2136.
13. Kim, J. H. and Y. S. Kim. 2001. Characterization of a metalloenzyme from a wild mushroom, *Tricholoma saponaceum*. *Biosci. Biotechnol. Biochem.* **65:** 356–362.
14. Kumar, C. G. and H. Takagi. 1999. Microbial alkaline proteases: From a bioindustrial viewpoint. *Biotechnol. Adv.* **17:** 561–594.
15. Laemmli, U. K. and M. Favre. 1973. Maturation of the head of bacteriophage T4. I. DNA packaging events. *J. Mol. Biol.* **80:** 575–599.
16. Lam, S. S., H. Wang, and T. B. Ng. 1998. Purification and characterization of novel ribosome inactivating proteins, alpha- and beta-pisavins, from seeds of the garden pea *Pisum sativum*. *Biochem. Biophys. Res. Commun.* **253:** 135–142.
17. Liu, M., J. Li, F. Kong, J. Lin, and Y. Gao. 1998. Induction of immunomodulating cytokines by a new polysaccharide–peptide complex from culture mycelia of *Lentinus edodes*. *Immunopharmacology* **40:** 187–198.
18. Mares-Guia, M. and E. Shaw. 1965. Studies on the active center of trypsin. The binding of amidines and guanidines as models of the substrate side chain. *J. Biol. Chem.* **240:** 1579–1585.
19. Matta, H. and V. Punj. 1998. Isolation and partial characterization of a thermostable extracellular protease of *Bacillus polymyxa* B-17. *Int. J. Food Microbiol.* **42:** 139–145.
20. Melgar, M. J., J. Alonso, and M. A. Garcia. 2009. Mercury in edible mushrooms and underlying soil: Bioconcentration factors and toxicological risk. *Sci. Total Environ.* **407:** 5328–5334.
21. Ng, T. B. 1998. A review of research on the protein-bound polysaccharide (polysaccharopeptide, psp) from the mushroom *Coriolus versicolor* (Basidiomycetes: Polyporaceae). *Gen. Pharmacol.* **30:** 1–4.
22. Nonaka, T., H. Ishikawa, Y. Tsumuraya, Y. Hashimoto, and N. Dohmae. 1995. Characterization of a thermostable lysine-specific metalloendopeptidase from the fruiting bodies of a basidiomycete, *Grifola frondosa*. *J. Biochem.* **118:** 1014–1020.
23. Palmieri, G., C. Bianco, G. Cennamo, P. Giardina, G. Marino, M. Monti, and G. Sannia. 2001. Purification, characterization, and functional role of a novel extracellular protease from *Pleurotus ostreatus*. *Appl. Environ. Microbiol.* **67:** 2754–2759.
24. Sesli, E., M. Tuzen, and M. Soylak. 2008. Evaluation of trace metal contents of some wild edible mushrooms from Black Sea region, Turkey. *J. Hazard. Mater.* **160:** 462–467.
25. Shaginian, K. A., I. A. Alekhina, and N. P. Denisova. 1990. Serine proteinase from the higher basidiomycetes of *Coprinus* genus. *Biokhimiia* **55:** 1387–1395.
26. Shen, M. H., J. S. Kim, K. Sapkota, S. E. Park, B. S. Choi, S. Kim, *et al*. 2007. Purification, characterization, and cloning of fibrinolytic metalloprotease from *Pleurotus ostreatus* mycelia. *J. Microbiol. Biotechnol.* **17:** 1271–1283.
27. Soponsathien, S. 1998. Some characteristics of ammonia fungi 1. In relation to their ligninolytic enzyme activities. *J. Gen. Appl. Microbiol.* **44:** 337–345.
28. Suzuki, M., S. Higuchi, Y. Taki, S. Taki, K. Miwa, and J. Hamuro. 1990. Induction of endogenous lymphokine-activated killer activity by combined administration of lentinan and interleukin 2. *Int. J. Immunopharmacol.* **12:** 613–623.
29. Wang, H. and T. B. Ng. 2006. Ganodermin, an antifungal protein from fruiting bodies of the medicinal mushroom *Ganoderma lucidum*. *Peptides* **27:** 27–30.
30. Wang, H., T. B. Ng, and Q. Liu. 2003. A novel lectin from the wild mushroom *Polyporus adusta*. *Biochem. Biophys. Res. Commun.* **307:** 535–539.
31. Wang, H. and T. B. Ng. 2001. Isolation and characterization of velutin, a novel low-molecular-weight ribosome-inactivating protein

from winter mushroom (*Flammulina velutipes*) fruiting bodies. *Life Sci.* **68:** 2151–2158.
32. Wang, H. and T. B. Ng. 2001. Pleureryn, a novel protease from fresh fruiting bodies of the edible mushroom *Pleurotus eryngii. Biochem. Biophys. Res. Commun.* **289:** 750–755.
33. Wang, H. X. and T. B. Ng. 2001. Isolation of pleuturegin, a novel ribosome-inactivating protein from fresh sclerotia of the edible mushroom *Pleurotus tuber-regium. Biochem. Biophys. Res. Commun.* **288:** 718–721.
34. Wang, H. X., W. K. Liu, T. B. Ng, V. E. Ooi, and S. T. Chang. 1995. Immunomodulatory and antitumor activities of a polysaccharide-peptide complex from a mycelial culture of *Tricholoma* sp., a local edible mushroom. *Life Sci.* **57:** 269–281.
35. Wang, H. X., T. B. Ng, W. K. Liu, V. E. Ooi, and S. T. Chang. 1996. Polysaccharide–peptide complexes from the cultured mycelia of the mushroom *Coriolus versicolor* and their culture medium activate mouse lymphocytes and macrophages. *Int. J. Biochem. Cell Biol.* **28:** 601–607.
36. Wong, J. H., C. C. Wong, and T. B. Ng. 2006. Purification and characterization of a galactose-specific lectin with mitogenic activity from pinto beans. *Biochim. Biophys. Acta* **1760:** 808–813.
37. Wu, D. M., W. Q. Duan, Y. Liu, and Y. Cen. 2010. Anti-inflammatory effect of the polysaccharides of golden needle mushroom in burned rats. *Int. J. Biol. Macromol.* **46:** 100–103.
38. Yaoita, Y., R. Kohata, R. Kakuda, K. Machida, and M. Kikuchi. 2002. Ceramide constituents from five mushrooms. *Chem. Pharm. Bull. (Tokyo)* **50:** 681–684.
39. Yaoita, Y., K. Matsuki, T. Iijima, S. Nakano, R. Kakuda, K. Machida, and M. Kikuchi. 2001. New sterols and triterpenoids from four edible mushrooms. *Chem. Pharm. Bull. (Tokyo)* **49:** 589–594.
40. Zhang, G. Q., J. Sun, H. X. Wang, and T. B. Ng. 2009. A novel lectin with antiproliferative activity from the medicinal mushroom *Pholiota adiposa. Acta Biochim. Pol.* **56:** 415–421.
41. Zhang, X. Q., Q. H. Liu, G. Q. Zhang, H. X. Wang, and T. B. Ng. 2010. Purification and molecular cloning of a serine protease from the mushroom *Hypsizigus marmoreus. Process. Biochemistry* **45:** 724–730.
42. Zheng, R., S. Jie, D. Hanchuan, and W. Moucheng. 2005. Characterization and immunomodulating activities of polysaccharide from *Lentinus edodes. Int. Immunopharmacol.* **5:** 811–820.