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A novel alkaline protease with antiproliferative activity from fresh fruiting bodies of the toxic wild mushroom *Amanita farinosa*

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A novel protease with a molecular mass of 15 kDa was purified from fresh fruiting bodies of the wild mushroom *Amanita farinosa*. The purification protocol entailed anion exchange chromatography on DEAE-cellulose, affinity chromatography on Affi-gel blue gel, cation exchange chromatography on SP-Sepharose, and gel filtration by fast protein liquid chromatography on Superdex 75. The protease was unadsorbed on DEAE-cellulose but adsorbed on Affi-gel blue gel and SP-Sepharose. It demonstrated a single 15-kDa band in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS/PAGE) and a 15-kDa peak in gel filtration. The optimal pH and optimal temperature of the protease were pH 8.0 and 65°C, respectively. Proliferation of human hepatoma HepG2 cells was inhibited by the protease with an IC_{50} of 25 μM. The protease did not have antifungal or ribonuclease activity.

Keywords: alkaline protease; *Amanita farinosa*; mushroom; fruiting bodies; antiproliferative; purification

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INTRODUCTION

Mushrooms are components of popular dishes in Oriental as well as Western cuisine. This is not only due to their delicious taste, but also because of their extensive nutritive value. Nowadays, with more and more people paying close attention to issues related to health, many investigators show interest in the highly nutritive mushrooms.

Mushrooms are abundant in proteins which have beneficial effects. To date, mushroom proteins have been purified and characterized including ribosome inactivating proteins (Wang & Ng, 2000a; Lam & Ng, 2001a, 2001b; Wang & Ng, 2001a; Ng & Wang, 2004a; 2004b), antifungal proteins (Lacadena *et al.*, 1995; Lam & Ng, 2001a; Wang & Ng, 2004; Guo *et al.*, 2005), ribonucleases (Wang & Ng, 2001c; Wang & Ng, 2003a, 2003b), ubiquitin-like proteins and peptides (Wang & Ng, 2000b; Wang *et al.*, 2003b), lectins (Wang *et al.*, 2002; Wang *et al.*, 2003a; Liu *et al.*, 2004), proteases (Sattar *et al.*, 1990; Nonaka *et al.*, 1995), cellulases (Teunissen & Op den Camp, 1993), xylanases (Kutasi *et al.*, 2001), laccases (Slomczynski *et al.*, 1995), invertases (Boddy *et al.*, 1993), and trehalose phosphorylases (Wannet *et al.*, 1998). Many of these proteins have applicable activities because of their functions like antiproliferative activity toward tumor cells (Wang *et al.*, 1995; Ngai & Ng, 2004), antitumor activity (Wang *et al.*, 2000; Sun *et al.*, 2010), mitogenic activity toward spleen cells (Wang *et al.*, 2002; Ngai & Ng, 2004), and inhibitory activity toward HIV-1 reverse transcriptase (Wang & Ng, 2001c).

The objective of this paper was to purify a protease from the wild mushroom *Amanita farinosa* and ascertain its characteristics to find out whether it has any potentially useful activities.

Many mushroom of the *Amanita* genus are toxic, e.g., *A. excelsa, A. farinosa, A. phalloides, A. pseudoporphyria, A. spissa, A. spreta* and *A spissacea*. Very few can be consumed without intoxication, e.g., *A. rubescens* and *A. viryenefides* are edible (Chang & Mao, 1995).

MATERIALS AND METHODS

Materials. Fruiting bodies of the mushroom *Amanita farinosa* were collected in the Yunnan Province in China. DEAE-cellulose was from Sigma Chemical Company, St. Louis, Missouri, USA. Affi-gel blue gel was from Bio-Rad, California, USA. SP-Sepharose and Superdex 75 HR 10/30 column were from GE Healthcare, Sweden. HepG2 cell line was purchased from American Type Culture Collection [ATCC], Manassas, USA. All other chemicals used were of analytical grade.

Isolation procedure. Fresh fruiting bodies of the wild mushroom *A. farinosa* (240 g) were extracted by homogenizing in distilled water (3 ml/g) at 4°C for 12 h. The homogenate was centrifuged at 14000 × *g* for 25 min at 4°C. The supernatant was chromatographed on a column of DEAE-cellulose (5 cm × 10 cm), which had previously been equilibrated and was then eluted with 10 mM Tris/HCl buffer (pH 7.4). After removal of the unadsorbed fraction D1, adsorbed materials were eluted with 0.2 M NaCl and then with 1 M NaCl in the same buffer to yield fractions D2 and D3, respectively. Fraction D1 with protease activity was next subjected to a column of Affi-gel blue gel (2.5 cm × 20 cm) in the same buffer. The column was eluted at a flow rate of 2 ml/min. After the first unadsorbed fraction had flowed through the column, adsorbed proteins were desorbed

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Abbreviations: FPLC, fast protein liquid chromatography; PMSF, phenylmethylsulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid.

using a linear (0–1 M) NaCl gradient. Protease activity found in the adsorbed fraction (B2) was further purified by chromatography on an SP-Sepharose column (2.5 × 20 cm) in 10 mM NaOAc buffer (pH 4.8). The active fraction (SP4) was eluted with a linear 0–1 M NaCl gradient. Fraction SP4 was subsequently fractionated on an FPLC-Superdex 75 HR10/30 column in 0.2 M NH_4HCO_3 buffer (pH 8.5) (Ngai & Ng, 2004).

SDS/polyacrylamide gel electrophoresis (SDS/PAGE). SDS/PAGE was carried out in accordance with the procedure of Laemmli (1970), using a 12% resolving gel and a 5% stacking gel. At the end of electrophoresis, the gel was stained with Coomassie Brilliant Blue.

Amino acid sequence analysis. The N-terminal sequence of the protease was determined by using a Hewlett-Packard HP G1000A Edman degradation unit and an HP 1000 HPLC System.

Assay for proteolytic activity of *Amanita farinosa* protease. The activity of the purified protease toward casein (Sigma) was assayed following the method of Wang and Ng (2001b). The concentration of casein was 1 mg/ml in 10 mM phosphate buffer (pH 7.2). The sample (20 µl) was incubated with 180 µl casein solution at 37 °C for 15 minutes. The reaction was terminated by addition of 400 µl of 5% trichloroacetic acid (TCA). Then the sample was centrifuged (12 000 × g, 5 min, 4 °C). Absorbance of the surpernatant was read at 280 nm against water as blank. 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 (Wang & Ng, 2001b).

Effect of pH on protease activity. The effect of pH in the range of 3 to 9 was tested. The buffers used to cover the pH range were 0.1 M NaOAc buffer (AA) (pH 3–4), 0.1 M Mes buffer (pH 4–7), and 0.1 M Hepes buffer (pH 7–9). *A. farinosa* protease was kept in buffer solutions at different pH values for 15 min at 37 °C. Then the protease activity was determined.

Effect of temperature on protease activity. Samples of the protease were kept at several different temperatures (20 °C to 100 °C at 10 °C intervals) for 15 min. The reaction was carried out at the optimum pH (pH 8). After that, protease activity was determined. The temperature at which enzyme activity was maximal was the optimum temperature (Lam & Ng, 2001b).

Tumor cell viability assay. HepG2 cells were cultured in RPMI medium supplemented with 10% (v/v) fetal bovine serum (FBS), 100 mg/l streptomycin, and 100 IU/ml penicillin, at 37 °C in a humidified atmosphere of 5% (v/v) CO_2. Cells were subsequently seeded into 96-well plates at a concentration of 2×10^3 cells/well, and incubated for 24 h. Different concentrations of the protease in 100 µl complete RPMI medium were then added to the wells and incubated for 72 h (Wang *et al.*, 1995). After that, MTT quantification assays were carried out to measure the cells' viability. Briefly, 20 µl of a 5 mg/ml solution of [3-[4,5-dimethythiazol-2-yl]-2,5-diphenyltetrazolium bromide] (MTT) in phosphate buffered saline was spiked into each well, and the plates were incubated for 4 h. The plates were then centrifuged at 2500 r.p.m. for 5 min. The supernatant was carefully removed, and 150 µl of dimethyl sulfoxide was added in each well to dissolve the MTT formazan at the bottom of the wells. After 10 min, the absorbance at 590 nm was measured with a microplate reader. PBS was added into wells instead of protease as control.

Assay of antifungal activity. The assay for antifungal activity toward *Rhizoctonia solani*, and other fungal species, *Sclerotinia sclerotiorum*, *R. cerealis*, and *Fusarium oxysporum*, was carried out in 90 × 15 mm Petri plates containing 10 ml of potato dextrose agar. After the mycelial colony had developed, sterile blank paper disks (0.625 cm in diameter) were placed at a distance of 0.5 cm away from the rim of the mycelial colony. An aliquot (15 µl) of the test sample was added to a disk. The plates were incubated at 23 °C for 72 h until mycelial growth had enveloped disks containing the control and had formed crescents of inhibition around disks containing samples with antifungal activity (Lacadena *et al.*, 1995).

Assay of ribonuclease activity. Yeast tRNA (Sigma) was used as substrate. Protease was incubated with 0.2 µg tRNA in 135 µl of 100 mM sodium phosphate buffer (pH 7.5) for 15 min. Then, 350 µl ice-cold 3.7% perchloric acid was added to terminate the reaction. After standing on ice for 15 min, the reaction mixture was centrifuged and the absorbance of the supernatant was read at 260 nm. One unit of ribonuclease activity is defined as the amount of enzyme that produces an absorbance increase of one per minute in the acid-soluble supernatant per milliter of reaction mixture under specified conditions (Wang & Ng, 2003a).

Assay for HIV-1 reverse transcriptase inhibitory activity. The inhibitory activity towards human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) was assessed by using an enzyme-linked immunosorbent assay (ELISA) kit from Boehringer Mannheim (Germany). The assay takes advantage of the ability of reverse transcriptase to synthesize DNA, starting from the template/primer hybrid poly(A)-oligo$(dT)_{15}$. Digoxigenin- and biotin-labeled nucleotides in an optimized ratio are incorporated into the DNA molecule synthesized by the RT. The detection and quantification of the synthesized DNA as a measure of RT activity follows sandwich ELISA protocol. A fixed amount (4–6 ng) of recombinant HIV-1 RT was used. The inhibitory activity of the protease was calculated as percent inhibition as compared to a control without the protein (Sun *et al.*, 2011).

RESULTS

Purification of *Amanita farinosa* protease

When the *Amanita farinosa* fruiting body extract was fractionated on DEAE-cellulose, the unadsorbed fraction D1 possessed protease activity whereas the adsorbed fractions D2 and D3 had no discernible activity. D1 was resolved by affinity chromatography on Affi-gel blue gel into an unadsorbed fraction B1 devoid of protease activity and an adsorbed fraction B2 with protease activity. When fraction B2 was chromatographed on SP- Sepharose, only the fourth fraction SP4 eluted by a linear 0–1 M NaCl gradient exhibited protease activity (Fig. 1). It was then separated by gel filtration on Superdex 75 into a smaller inactive fraction SU1 and a larger fraction SU2 with protease activity (Fig. 2). The molecular mass of SU2 was determined to be 15 kDa, as judged from its elution volume. This result was similar to that obtained from SDS/polyacrylamide gel electrophoresis (Fig. 3), in which the protease exhibited a single 15 kDa band. The yields and specific protease activities at the various stages of purification are shown in Table 1. The N-terminal amino acid sequence of the protease was

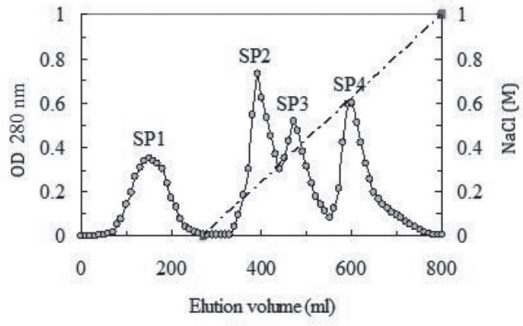

Figure 1. Ion exchange chromatography on SP-Sepharose.
Sample: fraction of *Amanita farinosa* fruiting body extract previously unadsorbed on DEAE-cellulose (fraction D1) and subsequently adsorbed on Affi-gel blue gel (fraction B2). Column dimensions: 2.5 cm×20 cm. Starting buffer: 10 mM NaOAc (pH 4.8). Slanting dotted line across the right half of the chromatogram represents a linear 0–1 M NaCl gradient used to elute adsorbed proteins. Protease activity was located in fraction SP4.

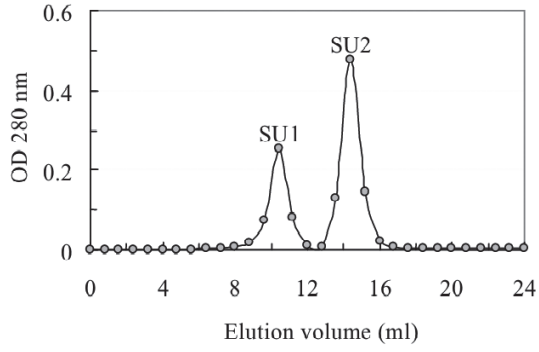

Figure 2. Gel filtration of fraction SP4 on a Superdex 75 HR 10/30 column by fast protein liquid chromatography.
Buffer: 0.2 M NH_4HCO_3 buffer (pH 8.5). Flow rate: 0.4 ml/min. Peak SU2 represents purified *Amanita farinosa* protease. Protease activity was located in fraction SU2.

VFDESAGQGT. It exhibited some similarity in N-terminal sequence to the protease from another Agaricales mushroom, *Pleurotus citrinopileatus*. However, there was little resemblance to a protease from *Agaricus bisporus*, also belonging to Agaricales (Table 2).

Characterization of *Amanita farinosa* protease

The pH dependence of the protease activity toward casein is shown in Fig. 4. The activity rose steadily when the pH was elevated from 3 to 8, and fell when the pH was raised beyond 8. The enzyme activity rose over the temperature range 20–65 °C (Fig. 5). The optimal pH and optimal temperature were pH 8 and 65 °C, respectively. Table 3 shows the viability of HepG2 cells after incubation with different concentrations of *Amanita farinosa* protease for 72 h. The inhibitory activity of the protease was dose-dependent. When the protease concentration was 100 μM, the inhibition ratio achieved was 63.9 %. The activity of purified protease was inhibited by PMSF (3 % residual activity with 1 mM PMSF), but not by EDTA or pepstatin A, which may be an indication that the enzyme belongs to the serine protease family. Antifungal, ribonuclease, HIV-1 reverse transcriptase inhibitory activities were undetectable for the protease (data not shown). Table 4 shows a comparison of *Amanita farinosa* protease and *P. citrinopileatus* protease. Both are monomeric alkaline proteases with similar

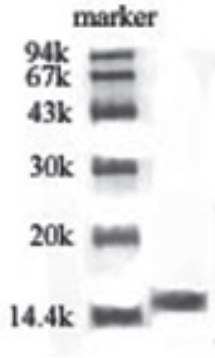

Figure 3. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis.
Right lane: *Amanita farinosa* protease (10 μg). **Left lane:** molecular mass standards, from top downward: phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa), and α-lactalbumin (14.4 kDa).

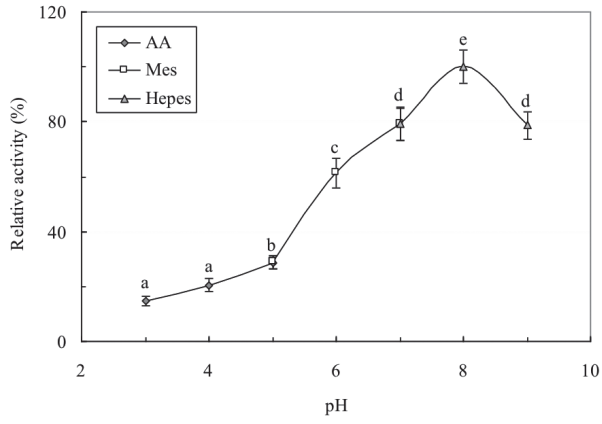

Figure 4. pH dependence of *Amanita farinosa* protease.
Temperature used: 37 °C, maximum activity = 100%. Results are presented as mean ±S.D. (n = 3). Different letters (a, b, c, etc) indicate statistically significant difference ($p < 0.05$) when the data are analyzed by analysis of variance followed by Duncan's multiple range test.

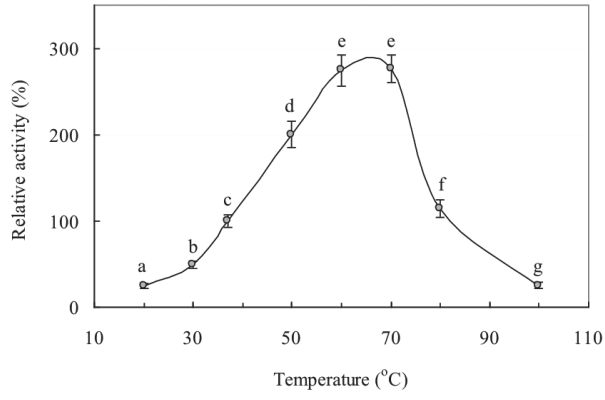

Figure 5. Effect of temperature on activity of *Amanita farinosa* protease.
Reaction time: 15 min. Substrate: casein. Buffer: 0.1 M Hepes buffer (pH 8.0). The activity at 37 °C used as reference was considered to be 100%. Results are presented as mean ±S.D. (n = 3). Different letters (a, b, c etc) indicate statistically significant difference ($p < 0.05$) when the data are analyzed by analysis of variance followed by Duncan's multiple range test.

Table 1. Yields and protease activities of various chromatographic fractions (from 240 g fresh fruiting bodies).

Chromatographic fraction	Yield (mg)	Specific protease activity (U/mg)	Purification fold
Extract	830.1	180.5	1
D1	241.6	437.7	2.4
D2	155.0	<10	–
D3	190.2	<10	–
B1	83.9	<10	–
B2	52.7	1422.0	7.9
B3	39.5	<10	–
SP1	8.7	<10	–
SP2	11.4	<10	–
SP3	8.2	231.3	1.3
SP4	9.3	5759.5	31.9
SU1	2.2	260.2	1.4
SU2	4.1	9200.0	51.0

Table 2. N-terminal sequence of *Amanita farinosa* protease compared to other fungal alkaline proteases (Results of BLAST search). Identical corresponding amino acid residues are underscored.

Species	Phylum	N-terminal sequence
Amanita farinosa	Basidiomycota	VFDESAGQGT
Pleurotus citrinopileatus	Basidiomycota	VCQCNAPWGL
Agaricus bisporus	Basidiomycota	MHFSLSFATL
Cordyceps chlamydosporia	Ascomycota	AIVEQQGAPW
Paecilomyces lilacinus	Ascomycota	ARAPLLTPRG
Penicillium chrysogenum	Ascomycota	MGFLKLLSTS
Aspergillus oryzae	Ascomycota	MQSIKRTLLL

Table 3. Inhibition of proliferation of HEPG2 cells by *Amanita farinosa* protease as determined by MTT assay.
PBS was used in the control instead of the protease.

Protease concentration (μM)	Inhibition of cell proliferation(%)
100	63.9
50	57.6
25	50.0
12.5	38.9
6.2	32.0
3.1	26.3
1.6	21.2

Each value is the mean for triplicate determinations, with an S.D. less than 10%.

chromatographic behavior on ion exchangers and slightly similar N-terminal amino acid sequences.

DISCUSSION

An alkaline protease with a molecular mass of 15 kDa was isolated from fresh fruiting bodies of the wild mushroom *Amanita farinosa*. Its N-terminal amino acid sequence was VFDESAGQGT. Compared with the reported proteases or metalloproteases from other mushrooms, its molecular mass is smaller than those from *Armillariella mellea* (Kim & Kim, 1999), *Grifola frondosa, Pleurotus ostreatus* (Nonaka *et al*., 1997), and *Tricholoma saponaceum* (Kim & Kim, 2001), which are in the range from 18.5 to 20 kDa. However, the molecular mass of the *A. farinosa* protease is a little larger than that of pleureryn from *Pleurotus eryngii*, which has a molecular mass 11.5 kDa (Kim & Kim, 2001).

The optimal pH of the protease from *A. farinosa* was pH 8. It indicated that this protease was an alkaline protease. When the pH was raised to 9, close to 80% of the activity of the protease was preserved. However, only about one-fifth of the activity was retained at pH 4. The activity of the *A. farinosa* protease reached the maximum at 65 °C and at pH 8. However, its activity showed a precipitous decline as the temperature was raised from 70 °C to 80 °C. Thus, it was a moderately thermostable protease. The *A. farinosa* protease exhibits a higher optimal pH and a higher optimal temperature than some reported mushroom proteases (Kim *et al*., 2006; Park *et al*., 2007; Shen *et al*., 2007).

It has long been known that chemical drugs can induce numerous side effects and cytotoxicity when used for the treatment of cancer. In an attempt to tackle this problem, scientists are now searching for materials with lower cytotoxicity and fewer side effects than chemical drugs. In fact, natural products such as those from mushrooms are good choices for replacement of toxic drugs as they are used widely in Chinese medicine. Abundant resources also exist in mushrooms which exhibit a multitude of biological activities (Nonaka *et al*., 1997).

It has been reported that many mushroom lectins display antiproliferative activities toward tumor cells. However, no antiproliferative activity of mushroom proteases has been reported. The isolated protease demonstrated an anti-tumor activity toward HepG2 cells with an IC_{50} of about 25 μM, which represents a fairly potent activity. It is well known that some proteases like caspases play a role in the apoptotic death of tumor cells (Puertollano *et al*., 2008). On the other hand, protease inhibitors like those of leguminous origin (Banerji *et al*., 1998) have antitumor activity.

A protease (Nygren *et al*., 2007), a protease inhibitor (Doljak *et al*., 2001), a hemolysin (Seeger, 1977), and a lectin (Zhuang *et al*., 1996) have been isolated from *Amanita* spp. No proteins have been purified from *Amanita farinosa*. The present study thus adds to the scanty literature. We have assayed the fruiting body extract of *A. farinosa* for hemagglutinating (lectin), antifungal and ribonuclease activities. The negative results indicate the absence of those bioactive proteins in *A. farinosa*.

Proteases are a class of enzymes with important applications in industrial fields, such as in the food, detergent, leather, pharmaceutical, and silk industries and for recovery of silver from used X-ray films (Sattar *et al*., 1990; Nonaka *et al*., 1995; Nygren *et al*., 2007). Thirty percent of the total enzymes produced in the world are proteases (in detergent industries). Besides industrial applications, proteases play important physiological roles in sporulation, conidial discharge, germination, enzyme modification, nutrition, protein turnover, and regulation of gene expression (Rao *et al*., 1998). The *A. farinosa* protease may have similar roles in the mushroom. In addition,

Table 4. Comparison of characteristics of proteases from *Amanita farinosa* and *Pleurotus citrinopileatus*.

	Amanita farinosa protease	*P. citrinopileatus* protease
Chromatographic behavior on anion exchangers	unadsorbed	unadsorbed
Chromatographic behavior on cation exchangers	adsorbed	adsorbed
Purification fold	51	30
Yield (mg/kg)	17	29
N-terminal sequence	VFDESAGQGT	VCQCNAPWGL
Molecular mass (kDa)	15	28
Mornomeric	Yes	Yes
Specific protease activity (u/mg)	9200	14526
Optimum pH	8	10
Optimum temperature (°C)	65	50
Anti-proliferative activity on hepatoma cells	IC_{50} = 25 μM	Not tested

Table 5. Influence of inhibitors on *A. farinose* protease.

Chemical	Concentration	Residual activity (%)
None		100%
PMSF	1 mM	3%
	0.2 mM	22%
	0.04 mM	70%
Pepstatin A	1 mM	88%
	0.2 mM	89%
	0.04 mM	100%
EDTA	1 mM	93%
	0.2 mM	96%
	0.04 mM	101%

Each residual activity is the mean for triplicate determinations, with an S.D. less than 10%.

the protease may act as a defense protein by hydrolyzing proteins of invading pathogens.

To recapitulate, an alkaline protease with relatively high thermostability and pH stability and potent antiproliferative activity against hematoma cells was isolated from a wild mushroom of which little is known.

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REFERENCE

Banerji A, Fernandes A, Bane S (1998) Treatment with field bean protease inhibitor can effectively repress ethylnitrosourea (ENU)-induced neoplasms of the nervous system in Sprague-Dawley rats. *Cancer Lett* **130:** 161–167.

Boddy LM, Berges T, Barreau C, Vainstein MH, Dobson MJ, Ballance DJ, Peberdy JF (1993) Purification and characterisation of an *Aspergillus niger* invertase and its DNA sequence. *Curr Genet* **24:** 60–66.

Chang ST, Mao XL (1995) Hong Kong Mushrooms. *Chinese University of Hong Kong Press*. Hong Kong.

Doljak B, Stegnar M, Urleb U, Kreft S, Umek A, Ciglaric M, Strukelj B, Popovic T (2001) Screening for selective thrombin inhibitors in mushrooms. *Blood Coagul Fibrinolysis* **12:** 123–128.

Guo Y, Wang H, Ng TB (2005) Isolation of trichogin, an antifungal protein from fresh fruiting bodies of the edible mushroom *Tricholoma giganteum*. *Peptides* **26:** 575–580.

Kim JH, Kim YS (1999) A fibrinolytic metalloprotease from the fruiting bodies of an edible mushroom, *Armillariella mellea*. *Biosci Biotechnol Biochem* **63:** 2130–2136.

Kim JH, Kim YS (2001) Characterization of a metalloenzyme from a wild mushroom, *Tricholoma saponaceum*. *Biosci Biotechnol Biochem* **65:** 356–362.

Kim JS, Sapkota K, Park SE, Choi BS, Kim S, Nguyen TH, Kim CS, Choi HS, Kim MK, Chun HS, Park Y, Kim SJ (2006) A fibrinolytic enzyme from the medicinal mushroom *Cordyceps militaris*. *J Microbiol* **44:** 622–631.

Kutasi J, Bata A, Brydl E, Rafai P, Jurkovich V (2001) Characterisation and effects of a xylanase enzyme preparation extracted from *Thermomyces lanuginosus* cultures. *Acta Vet Hung* **49:** 175–184.

Lacadena J, Martinez del Pozo A, Gasset M, Patino B, Campos-Olivas R, Vazquez C, Martinez-Ruiz A, Mancheno JM, Onaderra M, Gavilanes JG (1995) Characterization of the antifungal protein secreted by the mould *Aspergillus giganteus*. *Arch Biochem Biophys* **324:** 273–281.

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:** 680–685.

Lam SK, Ng TB (2001a) First simultaneous isolation of a ribosome inactivating protein and an antifungal protein from a mushroom (*Lyophyllum shimeji*) together with evidence for synergism of their antifungal effects. *Arch Biochem Biophys* **393:** 271–280.

Lam SK, Ng TB (2001b) Hypsin, a novel thermostable ribosome-inactivating protein with antifungal and antiproliferative activities from fruiting bodies of the edible mushroom *Hypsizigus marmoreus*. *Biochem Biophys Res Commun* **285:** 1071-1075.

Liu Q, Wang H, Ng TB (2004) Isolation and characterization of a novel lectin from the wild mushroom *Xerocomus spadiceus*. *Peptides* **25**: 7–10.

Ng TB, Wang H (2004a) Adustin, a small translation-inhibiting polypeptide from fruiting bodies of the wild mushroom *Polyporus adusta*. *Peptides* **25:** 689–692.

Ng TB, Wang HX (2004b) Flammin and velin: new ribosome inactivating polypeptides from the mushroom *Flammulina velutipes*. *Peptides* **25**: 929–933.

Ngai PH, Ng TB (2004) A mushroom (*Ganoderma capense*) lectin with spectacular thermostability, potent mitogenic activity on splenocytes, and antiproliferative activity toward tumor cells. *Biochem Biophys Res Commun* **314**: 988–993.

Nonaka T, Dohmae N, Hashimoto Y, Takio K (1997) Amino acid sequences of metalloendopeptidases specific for acyl-lysine bonds from *Grifola frondosa* and *Pleurotus ostreatus* fruiting bodies. *J Biol Chem* **272**: 30032–30039.

Nonaka T, Ishikawa H, Tsumuraya Y, Hashimoto Y, Dohmae N (1995) Characterization of a thermostable lysine-specific metalloendopeptidase from the fruiting bodies of a basidiomycete, *Grifola frondosa*. *J Biochem* **118:** 1014–1020.

Nygren CM, Edqvist J, Elfstrand M, Heller G, Taylor AF (2007) Detection of extracellular protease activity in different species and genera of ectomycorrhizal fungi. *Mycorrhiza* **17:** 241–248.

Park SE, Li MH, Kim JS, Sapkota K, Kim JE, Choi BS, Yoon YH, Lee JC, Lee HH, Kim CS, Kim SJ (2007) Purification and characterization of a fibrinolytic protease from a culture supernatant of *Flammulina velutipes* mycelia. *Biosci Biotechnol Biochem* **71:** 2214–2222.

Puertollano MA, Carrera MP, Puertollano E, Alvarez de Cienfuegos G, Ramirez-Exposito MJ, De Pablo MA, Martinez-Martos JM (2008) Analysis of caspase activities in rat mammary tumours induced by N-methyl-nitrosourea. *Oncol Rep* **20:** 657–662.

Rao GS, Arora S, Kataria S (1998) A proteinase K inhibitor using alpha,beta-unsaturated (dehydro) residues: a presumptive model. *J Biomol Struct Dyn* **15:** 1053–1058.

Sattar AK, Yamamoto N, Yoshimoto T, Tsuru D (1990) Purification and characterization of an extracellular prolyl endopeptidase from *Agaricus bisporus*. *J Biochem* **107:** 256–261.

Seeger R (1977) Hemolysins in mushrooms of the genus Amanita. *Curr Probl Clin Biochem* **7**: 15–21.

Shen MH, Kim JS, Sapkota K, Park SE, Choi BS, Kim S, Lee HH, Kim CS, Chun HS, Ryoo CI, Kim SJ (2007) Purification, characterization, and cloning of fibrinolytic metalloprotease from *Pleurotus ostreatus* mycelia. *J Microbiol Biotechnol* **17:** 1271–1283.

Slomczynski D, Nakas JP, Tanenbaum SW (1995) Production and Characterization of Laccase from *Botrytis cinerea* 61–34. *Appl Environ Microbiol* **61:** 907–912.

Sun J, Wang H, Ng TB (2010) Trypsin isoinhibitors with antiproliferative activity toward leukemia cells from *Phaseolus vulgaris* cv "White Cloud Bean". *J Biomed Biotechnol* **2010:** 219793.

Sun J, Wang HX, Ng TB (2011) Isolation of a laccase with HIV-1 reverse transcriptase inhibitory activity from fresh fruiting bodies of the *Lentinus edodes* (Shiitake mushroom). *Indian J Biochem Biophys* **48:** 88–94.

Teunissen MJ, Op den Camp HJ (1993) Anaerobic fungi and their cellulolytic and xylanolytic enzymes. *Antonie Leeuwenhoek* **63:** 63–76.

Wang H, Gao J, Ng TB (2000) A new lectin with highly potent antihepatoma and antisarcoma activities from the oyster mushroom *Pleurotus ostreatus*. *Biochem Biophys Res Commun* **275:** 810–816.

Wang H, Ng TB (2001a) 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.

Wang H, Ng TB (2001b) Pleureryn, a novel protease from fresh fruiting bodies of the edible mushroom *Pleurotus eryngii*. *Biochem Biophys Res Commun* **289:** 750–755.

Wang H, Ng TB (2003a) Isolation of a ribonuclease from fruiting bodies of the wild mushroom *Termitomyces globulus*. *Peptides* **24:** 973–977.

Wang H, Ng TB (2003b) A ribonuclease with distinctive features from the wild green-headed mushroom *Russulus virescens*. *Biochem Biophys Res Commun* **312:** 965–968.

Wang H, Ng TB (2004) Eryngin, a novel antifungal peptide from fruiting bodies of the edible mushroom *Pleurotus eryngii*. *Peptides* **25:** 1–5.

Wang H, Ng TB, Liu Q (2002) Isolation of a new heterodimeric lectin with mitogenic activity from fruiting bodies of the mushroom *Agrocybe cylindracea*. *Life Sci* **70:** 877–885.

Wang H, Ng TB, Liu Q (2003a) A novel lectin from the wild mushroom *Polyporus adusta*. *Biochem Biophys Res Commun* **307:** 535–539.

Wang HX, Ng TB (2000a) Flammulin: a novel ribosome-inactivating protein from fruiting bodies of the winter mushroom *Flammulina velutipes*. *Biochem Cell Biol* **78:** 699–702.

Wang HX, Ng TB (2000b) Isolation of a novel ubiquitin-like protein from *Pleurotus ostreatus* mushroom with anti-human immunodeficiency virus, translation-inhibitory, and ribonuclease activities. *Biochem Biophys Res Commun* **276:** 587–593.

Wang HX, Ng TB (2001c) Purification and characterization of a potent homodimeric guanine-specific ribonuclease from fresh mushroom (*Pleurotus tuber-regium*) sclerotia. *Int J Biochem Cell Biol* **33**: 483–490.

Wang HX, Ng TB, Liu WK, Ooi VE, Chang ST (1995) Isolation and characterization of two distinct lectins with antiproliferative activity from the cultured mycelium of the edible mushroom *Tricholoma mongolicum*. *Int J Pept Protein Res* **46**: 508–513.

Wang HX, Ngai HK, Ng TB (2003b) A ubiquitin-like peptide with ribonuclease activity against various polyhomoribonucleotides from the yellow mushroom *Cantharellus cibarius*. *Peptides* **24:** 509–513.

Wannet WJ, Op den Camp HJ, Wisselink HW, van der Drift C, Van Griensven LJ, Vogels GD (1998) Purification and characterization of trehalose phosphorylase from the commercial mushroom *Agaricus bisporus*. *Biochim Biophys Acta* **1425:** 177–188.

Zhuang C, Murata T, Usui T, Kawagishi H, Kobayashi K (1996) Purification and characterization of a lectin from the toxic mushroom *Amanita pantherina*. *Biochim Biophys Acta* **1291:** 40–44.