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An Antiproliferative Ribonuclease from Fruiting Bodies of the Wild Mushroom *Russula delica*

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An antiproliferative ribonuclease with a new N-terminal sequence was purified from fruiting bodies of the edible wild mushroom *Russula delica* in this study. This novel ribonuclease was unadsorbed on DEAE-cellulose, but absorbed on SP-Sepharose and Q-Sepharose. It had a molecular mass of 14 kDa, as judged by fast protein liquid chromatography on Superdex 75 and SDS–polyacrylamide gel electrophoresis. Its optimal pH and optimal temperature were pH 5 and 60°C, respectively. The ranking of its activity toward various polyhomoribonucleotides was poly C> poly G>poly A>poly U. It could inhibit proliferation of HepG2 and MCF-7 cancer cells with an IC_{50} value of 8.6 μM and 7.2 μM, respectively. It was devoid of antifungal and HIV-1 reverse transcriptase inhibitory activity.

Keywords: Ribonuclease, *Russula delica*, antiproliferative, purification

Ribonucleases (RNases) belong to an important family of proteins that have been studied intensively. They are found in abundance in plants [8], fungi [20, 22, 28, 36], bacteria [19], and various mammalian organs including the brain, kidney [13], pancreas, and liver [11]. RNases isolated from different tissues may have different structures [11, 13, 25] and can exhibit different activities such as antineoplastic [1], antitumor [9], immunosuppressive [16, 21], antiproliferative [21], antiviral, and antifungal [30] activities.

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The mushroom family Russulaceae is composed of two genera, *Russula* and *Lactarius*, the former being the majority. To date, only a ribonuclease [34] and a protein with anti-HIV-1 reverse transcriptase activity [38] have been isolated from mushrooms of the genus *Russula*. Only four reports on *Lactarius* lectin [6, 10, 24, 26] and one report on a *Lactarius* enzyme [17] are available. *Russula delica* is a wild mushroom on which few literatures have been reported before. Currently, there are only four publications concerning *Russula delica* in the literature. One of them is about the inefficient photosynthesis in the Mediterranean orchid *Limodorum abortivum*, which is caused by association with *Russula delica* [7]. Another is on the isolation of a new norsesquiterpenoid and three known sesquiterpenoids from the fruiting bodies of the mushroom [4]. The third paper is a study of the fatty acid content in spores of higher basidiomycete fungi including *Russula delica* as a method for chemotaxonomical classification of fungi [3]. The fourth paper is on the antioxidant activity of its extract [5].

The aim of this investigation was to isolate a ribonuclease from its fruiting bodies as well as to ascertain if it exhibits characteristics different from those of the previously reported mushroom ribonucleases [8, 21, 23, 35]. The present report is an addition to the meager literature on the mushroom *Russula delica*.

Materials and Methods

Materials

Fresh fruiting bodies of wild mushroom *Russula delica* were collected in Yunnan Province, China. DEAE-cellulose, SP-Sepharose, and Q-Sepharose were obtained from Sigma. The cell lines MCF-7 (breast

adenocarcinoma) and HepG2 (hepatoma) were purchased from American Type Culture Collection, U.S.A. All other chemicals used were of analytical grade.

Isolation Procedure

Fresh fruiting bodies of wild mushroom *Russula delica* (800 g) were extracted by homogenizing in distilled water (3 ml/g) and the homogenate was stirred overnight at 4°C before centrifugation. Following centrifugation of the extract at 14,000 ×*g* for 25 min, the supernatant was collected. Tris-HCl buffer (pH 7.4, 1.0 M) was added to the supernatant to bring the Tris concentration to 10 mM. The mixture was then chromatographed on a 5×20 cm column of DEAE-cellulose (Sigma). After removal of the unadsorbed fraction D1, adsorbed materials were eluted sequentially with 0.2 M NaCl and 1 M NaCl in the same buffer to yield fractions D2 and D3, respectively. Fraction D1 with ribonuclease activity was next subjected to chromatography on a 2.5×20 cm column of SP-Sepharose (GE Healthcare) in 10 mM NH_4OAc buffer (pH 5.0). Unadsorbed proteins (fraction SP1) were eluted with 10 mM NH_4OAc buffer, and adsorbed proteins were eluted using a linear (0–1 M) NaCl in the same buffer to yield 3 fractions: SP2, SP3, and SP4. Fraction SP3 was chromatographed on a 2.5×20 cm Q-Sepharose (GE Healthcare) column in 10 mM NH_4HCO_3 buffer (pH 9.4). Unadsorbed materials (fraction Q1) were eluted with the buffer, whereas adsorbed proteins (Q2 and Q3) were desorbed using a linear 0–1 M NaCl gradient. Ribonuclease activity found in fraction Q2 was further purified by fast protein liquid chromatography on a Superdex 75 HR 10/30 column (GE Healthcare) in 200 mM NH_4HCO_3 (pH 8.5) using an AKTA Purifier (GE Healthcare) [21].

SDS–Polyacrylamide Gel Electrophoresis (SDS–PAGE)

SDS–PAGE was carried out in accordance with the procedure of Laemmli and Favre [14], using a 12% resolving gel and a 5% stacking gel. After electrophoresis, the gel was stained with Coomassie brilliant blue.

Amino Acid Sequence Analysis

The N-terminal sequence of the ribonuclease was determined using a Hewlett-Packard HP G1000A Edman degradation unit and an HP 1000 HPLC system [27].

Assay for Ribonucleolytic Activity of *Russula delica* Ribonuclease

The activity of the purified RNase toward yeast tRNA (Sigma) was assayed by determining the generation of acid-soluble, UV-absorbing species using the method employed by Mock and Ng [18]. RNase was incubated with 10 µl of tRNA (10 mg/ml) and 5 µl of sample in 135 µl of 100 mM NaOAC buffer (pH 5.0) at 37°C for 15 min. The reaction was terminated by the use of 350 µl of ice-cold 3.4% perchloric acid. The sample was then centrifuged (12,000 ×*g*, 5 min) and the A 260 nm of the supernatant was read. One unit of RNase activity is defined as the amount of RNase that brings about an increase of 1 in A 260 nm per minute in the acid-soluble fraction per milliliter of reaction mixture under the specified conditions [31].

Effect of Temperature on Ribonuclease Activity

The ribonuclease was kept at various temperatures (from 20°C to 100°C, at 10°C intervals) for 15 min and its ribonuclease activity was determined after cooling down to room temperature [32].

Effect of pH on Ribonuclease Activity

The ribonuclease sample was kept in buffers with different pH values (from pH 2.2 to pH 10.0) instead of the aforementioned NaOAC buffer (pH 5.0), and the ribonuclease activity was analyzed by determining A 260 nm, as described above. The activity at 37°C was considered as 100% [34].

Activity of RNase Toward Polyhomoribonucleotides

The ribonucleolytic activity of the RNase toward polyhomoribonucleotides was determined with a modified method of Wang and Ng [31]. Incubation of the RNase with 100 µg of poly A, poly C, poly G, or poly U in 250 µl of 100 mM sodium acetate (pH 5.0) was carried out at 37°C for 1 h prior to the addition of 250 µl of ice-cold 1.2 M perchloric acid containing 20 mM lanthanum nitrate to terminate the reaction. After standing on ice for 15 min, the sample was centrifuged at 15,000 ×*g* for 15 min at 4°C. The absorbance of the supernatant, after appropriate dilution, was read at 260 nm (in the case of poly A, poly G, and poly U) or at 280 nm (in the case of poly C).

Assay of Antiproliferative Activity on Tumor Cell Lines

This assay was conducted in view of the report that some ribonucleases display antiproliferative activity on tumor cell lines [2]. The antiproliferative activity of the purified ribonuclease was determined as follows. MCF-7 (breast adenocarcinoma) and HepG2 (hepatoma) cell lines were cultured in the RPMI medium supplemented with 10% (v/v) fetal bovine serum (FBS), 100 mg/l streptomycin, and 100 IU/ml penicillin at 37°C and in a humidified atmosphere of 5% (v/v) CO_2. Cells were subsequently seeded onto 96-well plates at a concentration of 2×10^3 cells/well, and incubated for 24 h before the addition of the enzyme. Incubation was then carried out for another 48 h. Following that, the MTT assay was carried out to measure the cell viability. Briefly, 20 µl of a 5 mg/ml solution of MTT in phosphate-buffered saline was spiked into each well and the plates were incubated for 4 h. The plates were then centrifuged at 2,500 rpm for 5 min. The supernatant was carefully removed and 150 µl of dimethyl sulfoxide was added into each well to dissolve the MTT formazan at the bottom of the wells. After 10 min, the absorbance at 590 nm was measured using a microplate reader. PBS was added into the wells, in place of the ribonuclease, as a negative control [9].

Assay for HIV Reverse Transcriptase (HIV RT) Inhibitory Activity

The RNase was assayed for HIV RT inhibitory activity in view of reports that some RNases exhibit this activity [29]. The assay was performed, as described by Zheng *et al*. [41], using a nonradioactive enzyme-linked immunosorbent assay (ELISA) kit from Boehringer–Mannheim (Germany). The assay was carried out as stated in the protocol that came with the kit, except that each well contained 2 ng of recombinant HIV-1 reverse transcriptase in a total reaction volume of 60 µl. It made use of the ability of reverse transcriptase to synthesize DNA, starting from the template/primer hybrid poly(A)-oligo(dT) 15. In this assay, nucleotides, labeled with digoxigenin and biotin in an optimized proportion, were incorporated into the DNA freshly synthesized by the reverse transcriptase (RT). The detection and quantification of synthesized DNA as a parameter for RT activity follows a sandwich ELISA protocol. The surface of the microtiter plate modules, which had been coated with streptavidin, allowed the binding of biotin-labeled DNA. An antibody to digoxigenin (anti-DIG-POD), which had been conjugated to peroxidase, was

then used to bind to the digoxigenin-labeled DNA. This was followed by the addition of a peroxidase substrate. The peroxidase enzyme, conjugated to the antibody, then catalyzes the cleavage of the substrate to produce a colored product. The absorbance was then measured at 405 nm with a microtiter (ELISA) reader and then correlated to the level of RT activity. A fixed amount (4–6 ng) of recombinant HIV-1 reverse transcriptase was used, and the activity of inhibition exhibited by *Russula delica* RNase was determined as compared with a control without RNase.

Assay of Ability to Inhibit HIV-1 Integrase

Expression and purification of recombinant HIV-1 integrase. The plasmid that expressed His-tagged wild-type HIV-1 integrase, pT7-7-His (Y|TX)-HIV-1-IN, was a generous gift from Professor S.A. Chow (School of Medicine, UCLA). In order to express the protein, a 1-l culture of *E. coli* BL21 (DE3) cells containing the expression plasmid was grown at 37°C until A_{600} was 0.7–0.8. The cells were induced by addition of 0.8 mM IPTG (isopropyl-β-D-thiogalactopyranoside) and harvested, by centrifugation at 6,000 ×*g* for 10 min at 4°C, after 4 h of incubation. The cells were then suspended at a concentration of 10 g/ml wet cell paste in 20 mM Tris-HCl buffer (pH 8.0) containing 0.1 mM EDTA, 2 mM β-mercaptoethanol, 0.5 M NaCl, and 5 mM imidazole. Lysozyme was added to a final concentration of 0.2 mg/ml. After incubation at 4°C for 1 h, the lysate was sonicated and centrifuged at 40,000 ×*g* at 4°C for 20 min. The pellet was homogenized in 50 ml of buffer A (20 mM Tris-HCl, pH 8.0, 2 M NaCl, 2 mM β-mercaptoethanol) containing 5 mM imidazole. The suspension was then rotated at 4°C for 1 h, and cleared by centrifugation at 40,000 ×*g* at 4°C for 20 min. Thereafter, the supernatant was loaded onto a 1-ml chelating Sepharose (GE Healthcare) column charged with 50 mM imidazole. The column was then washed with five column volumes of buffer A containing 5 mM imidazole before the protein was eluted with three column volumes of buffer A containing 200 and 400 mM imidazole, respectively. Protein-containing fractions were pooled, and EDTA was added to a final concentration of 5 mM. The protein was first dialyzed against buffer B (20 mM HEPES, pH 7.5, 1 mM EDTA, 1 M NaCl, 20% glycerol) containing 2 mM β-mercaptoethanol, and then against buffer B containing 1 mM dithiothreitol. Aliquots of the protein were stored at −70°C [17].

HIV-1 integrase assay. A nonradioactive ELISA-based HIV-1 integrase assay was performed according to the DNA-coated plate method. In this study, 1 μg of *Sma*ll-linearized pBluescript SK was coated onto each well, in the presence of 2 M NaCl, as target DNA. The donor DNA was prepared by annealing VU5BR (5'-biotin-GTGTGGAAAATCTCTA- GCAGT-3') and VU5 (5'-ACTGCTAGA GATTTTCCACAC-3') in 10 mM Tris-HC1, pH 8.0, 1 mM EDTA, and 0.1 M NaCl at 80°C and then at room temperature for 30 min. The integrase reaction was performed in 20 mM HEPES (pH 7.5) containing 10 mM $MnCl_2$, 30 mM NaCl, 10 mM dithiothreitol, and 0.05% Nonidet-P40 (Sigma). After the integrase reaction, the biotinylated DNA immobilized on the wells was detected by incubation with streptavidin-conjugated alkaline phosphatase (Boehringer–Mannheim, Mannheim, Germany), followed by colorimetric detection with 1 mg/ml *p*-nitrophenyl phosphate in 10% diethanolamine buffer (pH 9.8) containing 0.5 mM $MgCl_2$. The absorbance due to the alkaline phosphatase reaction was measured at 415 nm and the ribosome inactivating protein trichosanthin was used as a positive control [17].

Screening for Inhibitory Effect on SARS Coronavirus Protease

The activity of SARS coronavirus (CoV) protease was indicated by the cleavage of a designed substrate, which was composed of two proteins linked by a cleavage site for SARS CoV protease. The reaction was performed in a mixture containing 5 mM SARS CoV protease, 5 mM sample, 20 mM substrate, and buffer [20 mM Tris-HCl (pH 7.5), 20 mM NaCl, and 10 mM beta-mercaptoethanol] for 40 min at 37°C. After 40 min, the reaction was stopped by heating at 100°C for 2 min. The reaction mixture was then analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. If SARS CoV protease is inhibited by the test sample, only one band belonging to the intact substrate will be observed from the SDS–PAGE analysis [12].

Assay of Antifungal Activity

The RNase was assayed for antifungal activity in view of reports that some RNases can exhibit such an activity [2, 15]. The assay for antifungal activity towards *Fusarium oxysporum*, *Mycosphaerella arachidicola*, and *Botrytis cinerea* 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 discs (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 ribonuclease was then added to a disc and the plates were incubated at 23°C for 72 h until mycelial growth had enveloped the discs containing the buffer control and had formed crescents of inhibition around discs containing samples with antifungal activity. An antifungal protein from lima beans was used as the positive control [39].

RESULTS

Purification of *Russula delica* Ribonuclease

When the *Russula delica* extract was fractionated on DEAE-cellulose, only the unadsorbed fraction D1, which was also the smallest unadsorbed fraction, possessed RNase activity. D1 was separated on SP-Sepharose into a small inactive unadsorbed fraction SP1 and three adsorbed

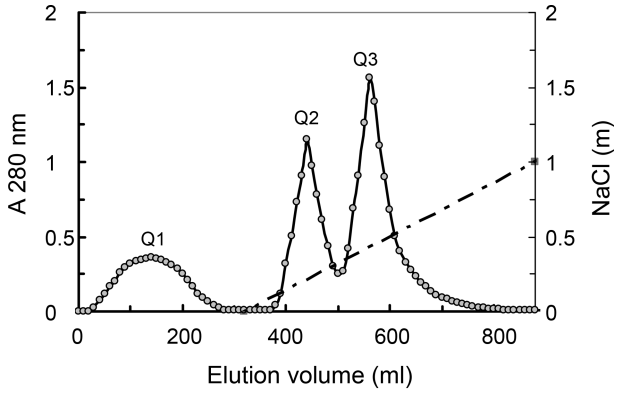

Fig. 1. Ion-exchange chromatography of fraction SP3 on a Q-Sepharose column (2.5×20 cm).
Starting buffer: 10 mM NH_4HCO_3 (pH 9.4). Slanting dotted line across the right side of the chromatogram represents the linear 0–1 M NaCl gradient used to elute adsorbed proteins.

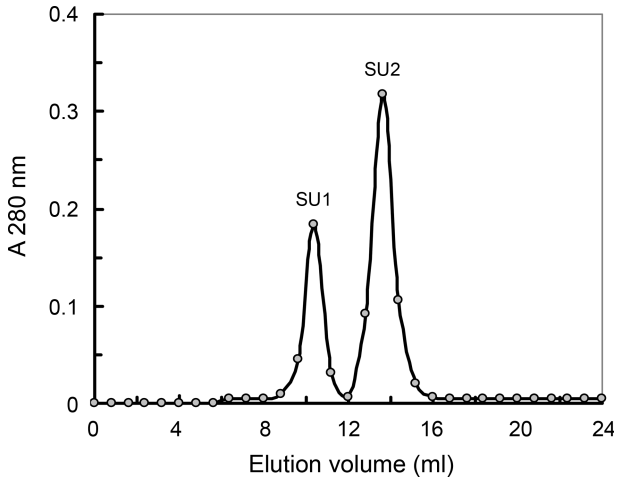

Fig. 2. Gel filtration of Q3 on a Superdex 75 HR 10/30 column by fast protein liquid chromatography.
Buffer: 0.2 M NH_4HCO_3 buffer (pH 8.5). Flow rate: 0.4 ml/min. Fraction SU2 represents purified ribonuclease.

fractions SP2, SP3, and SP4. Fraction SP3, which was the smallest of the adsorbed peaks, contained the bulk of the RNase activity. When SP3 was chromatographed on Q-Sepharose, the first adsorbed and also the largest fraction Q2, which was eluted with a linear 0–1 M NaCl gradient, exhibited RNase activity (Fig. 1). Q2 was separated on Superdex 75 into 2 fractions, SU1 and SU2. RNase activity was found to be enriched in the larger fraction SU2 (Fig. 2). The molecular mass of SU2 was judged, based on its elution volume, to be 14 kDa. In addition, SU2 also demonstrated a single 14-kDa band in SDS–PAGE (Fig. 3). The yields and specific ribonuclease activities at the various stages of purification are shown in Table 1. The N-terminal sequence of *Russula delica* ribonuclease was

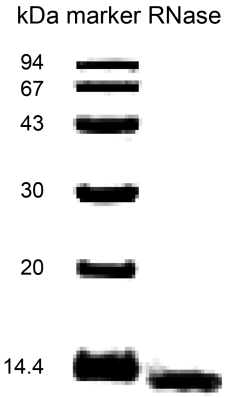

Fig. 3. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis.
Left lane: molecular mass standards from GE Healthcare; from top downward: phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa), and α-lactalbumin, (14.4 kDa). Right lane: *Russula delica* ribonuclease (9 μg).

Table 1. Yields and ribonuclease (RNase) activities of various chromatographic fractions derived from *Russula delica* fruiting body extract (from 800 g of fresh fruiting bodies).

Fraction with RNase activity	Yield (mg)	Specific RNase activity (U/mg)	Purification fold
Extract	2,488.2	86.8	1
D1	458.5	324.7	3.7
SP3	64.6	1,410.2	16.2
Q2	14.6	4,158.0	47.9
SU2	6.5	6,200.1	71.4

GCGATACKQV, which showed only slight similarity to those of the ribonucleases from *Lentinus edodes* and *Irpex lacteus* (Table 2).

Characterization of Ribonuclease Activity of Russula Delica RNase

As shown in Fig. 4, the enzyme activity was found to increase over the temperature range of 20–60°C. The activity at 20°C was less than 25% of that at 37°C, and similar to the activity at 100°C, whereas the activity at 60°C was 200% of that at 37°C. The pH dependence of the ribonucleolytic activity of the ribonuclease towards yeast tRNA is shown in Fig. 5. The activity rose sharply from pH 3 to 5 and declined steadily from pH 5 to 8. At pH 3 and pH 8, activity was undetectable. The optimal pH and temperature were found to be pH 5 and 60°C, respectively. The RNase exerted a ribonucleolytic activity of 0.8±0.1, 36.9±1.9, 0.6±0.1, and 3.6±0.2 U/mg (n=3) toward poly A, poly C, poly U, and poly G, respectively.

Other Biological Activities of *Russula delica* RNase

Proliferation of the cancer cell lines HepG2 and MCF-7 was inhibited by the RNase, with IC_{50} values of 8.6± 0.7 μM and 7.2±0.6 μM (mean±SD, n=3), respectively

Table 2. N-terminal sequence of *Russula delica* (RD) ribonuclease in comparison with ribonucleases from *Clitocybe maxima* (CM), *Lentinus edodes* (LE), *Irpex lacteus* (IL), *Dictyophora indusiata* (DI), *Pleurotus tuber-regium* (PT).

	N-terminal sequence	Reference
RD:	**GCG**A**T**ACKQV	This paper
LE:	ISS**GCG**T**T**GALSCSSNAKGTCCFEAPGGLI	Kobayashi et al., 2003
IL:	VNS**GCG**TSGAESCSNSDDGTCCFEAPGGLL	Watanabe et al., 1995
CM:	ETAHTHAGIQYSTVDVNNSIMKAVGGGAGN	Wang and Ng, 2004
DI:	GQPRQPQPQLLV	Wang and Ng, 2003
PT:	ALTAQDNRVRVGNRIVGNNFNFAAVQAAYY	Wang and Ng, 2001

Identical amino acid residues are underlined.

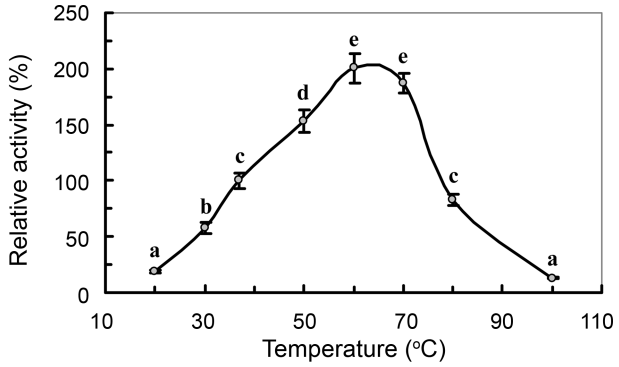

Fig. 4. Effect of temperature on the ribonucleolytic activity of *Russula delica* ribonuclease.
Reaction time: 15 min. Substrate: yeast tRNA. Buffer: 0.1 M NaOAC buffer (pH 5.0). Activity at 37°C was used as 100%. Results represent mean±SD (n=3). Different letters (a, b, c, d) next to the data points indicate statistically significant difference ($p<0.05$) when the data are analyzed by analysis of variance followed by Duncan's multiple range test.

(Table 3). The RNase, tested up to 100 μM, was devoid of antifungal, HIV-1 reverse transcriptase inhibitory, HIV-1 integrase inhibitory (Table 4), and SARS coronavirus protease inhibitory activities.

Comparison with Selected Mushroom RNases

Russula delica RNase had a molecular weight close to that of *Clitocybe maxima* RNase. Its chromatographic behavior on ion-exchange RNase was similar to that of *Russula virescens* RNase and it had a much higher specific RNase activity than other RNases. Its optimal pH was similar to that of *Russula virescens* RNase and other mushroom RNases. Its polyhomoribonucleotide specificity was similar to that of *Russula virescens* RNase. On the whole, *Russula delica* RNase was similar to its counterpart from *Russula virescens* to some extent (Table 5).

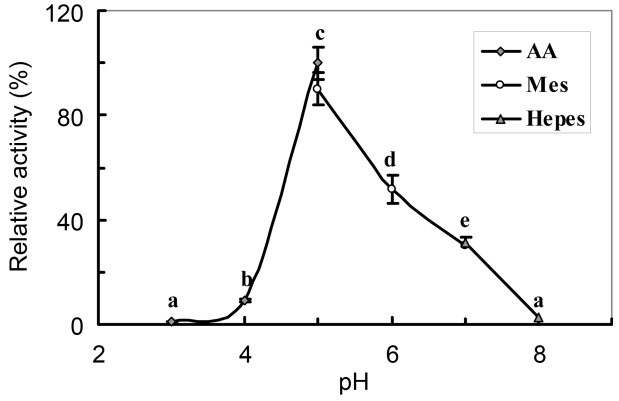

Fig. 5. pH dependence of *Russula delica* ribonuclease.
Temperature used: 37°C; maximum activity = 100%. Results represent mean±SD (n=3). Different letterss (a, b, c, d) next to the data points indicate statistically significant difference ($p<0.05$) when the data are analyzed by analysis of variance followed by Duncan's multiple range test.

Table 3. Dose-dependent inhibitory activity of *Russula delica* RNase toward HepG2 cells and MCF7 cells.

	20 μM	10 μM	5 μM	2.5 μM	IC_{50} (μM)
HepG2	75.7±5.2	54.3±3.6	32.6±2.1	15.8±1.3	8.6±0.7
MCF7	80.1±6.0	59.8±4.7	38.1±2.6	17.9±1.5	7.2±0.6

Results represent % inhibition (mean±SD, n=3).

DISCUSSION

The purification of a ribonuclease with antiproliferative activity from a wild mushroom is reported herein. Its chromatographic behavior is similar to those of RNases from *Pleurotus sajor-caju* [21] and *Pleurotus tuber-regium* [31]. The molecular mass of this ribonuclease (14 kDa) is within the range (9–42 kDa) reported for mushroom RNases and is similar to that of the ribonucleases from black oyster mushroom [32], *Pleurotus pulmonarius* [40], and portabella mushroom [37]. Its RNase activity towards yeast transfer RNA is much more potent than those of previously reported mushroom RNases (Table 5). Besides that, its optimum pH and temperature are similar to those of portabella mushroom RNase [37], *Hypsizigus marmoreus* RNase [9], and *Pleurotus sajor-caju* RNase [14]. The N-terminal sequence of *Russula delica* RNase reveals that it is only slightly similar to RNases from *Lentinus edodes* and *Irpex lacteus*, and distinctly different from those of other mushroom RNases. However, the aforementioned characteristics and also the N-terminal sequence of *Russula delica* RNase are different from those of other mushroom RNases reported so far, indicating that it is a novel RNase. The antiproliferative activity of *Russula delica* RNase is notable because most of the other mushroom RNases have not been assayed for this activity. Although, some ribonucleases from mushrooms exhibited antiproliferative, antifungal, and HIV-1 reverse transcriptase inhibitory activities [15, 29], *Russula delica* RNase is found to possess only antiproliferative activity and is devoid of the other activities. In addition, it also lacks HIV-1 integrase inhibitory and SARS coronavirus protease inhibitory activities. Thus, the ribonuclease isolated may play a role as a defense protein that hydrolyzes the RNA of invading pathogens.

When compared with *Russula virescens* RNase, *Russula delica* RNase shows similar chromatographic behavior on

Table 4. Inhibition rate (%) of isolated RNase on HIV-1 reverse transcriptase (RT) and HIV-1 integrase (IN).

Dose (μM)	HIV-1 RT	HIV-1 IN
12.5	4.8±0.5	4.9±0.4
25	3.5±0.4	5.1±0.6
50	3.7±0.3	4.2±0.5
100	2.6±0.3	3.4±0.3

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

Table 5. Comprison of biochemical characteristics and activities of ribonucleases from *Russula delica*, *Russula virescens*, *Clitocybe maxima*, *Dictyophora indusiate*, *Ganoderma lucidum*, and *Volvariella volvacea.*

	Russula delica	*Russula virescens*	*Clitocybe maxima*	*Dictyophora indusiata*	*Ganoderma lucidum*	*Volvariella volvacea*
Molecular mass (kDa)	14	28	17.5	28	43	42.5
Chromatographic behavior on						
(i) DEAE-cellulose	Unadsorbed	Adsorbed	Unadsorbed	Adsorbed	Adsorbed	Unadsorbed
(ii) Affi-gel Blue gel	-	-	Adsorbed	-	-	Adsorbed
(iii) CM-Sepharose	-	Adsorbed	Adsorbed	Adsorbed	Unadsorbed	Adsorbed
(iv) Q-Sepharose	Adsorbed	Adsorbed	-	Adsorbed	Adsorbed	-
(v) SP-Sepharose	Adsorbed	-	-	-	-	-
Specific RNase activity	6,200	216.9	1,173	564	180.6	500
Optimum pH	5.0	4.5	6.5–7.0	4.0–4.5	4.0	6.5–7.5
Optimum temperature	60°C	60°C	70°C	60°C	60°C	-
Activity toward polyhomoribonucleotides	poly C> poly G> poly A> poly U	poly C> poly A> poly U> poly G	poly A> poly G> poly U> poly C	poly U> poly A> poly G> poly C	poly U> poly A> poly C> poly G	poly U> poly C> poly G> poly A
Antiproliferative activity (IC_{50}, in μM)						
Hep G2	8.6	-	-	-	-	-
MCF 7	7.2	-	-	-	-	-

- Not determined or not attempted.

ion exchangers, optimum pH, optimum temperature, and polyhomoribonucleotide specificity. However, there are marked differences in specific RNase activity, molecular weight, and N-terminal sequence, indicating that these two RNases are distinct proteins.

In conclusion, *Russula delica* RNase possesses a unique N-terminal sequence, a high specific RNase activity, and an antiproliferative activity towards tumor cells.

Acknowledgments

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References

1. Adinolfi, B. S., V. Cafaro, G. Dalessio, and A. Didonato. 1995. Full antitumor action of recombinant seminal ribonuclease depends on the removal of its N-terminal methionine. *Biochem. Biophys. Res. Commun.* **213:** 525–532.
2. Ardelt, W., K. Shogen, and Z. Darzynkiewicz. 2008. Onconase and amphinase, the antitumor ribonucleases from *Rana pipiens* oocytes. *Curr. Pharm. Biotechnol*. **9:** 215–225.
3. Brondz, I., K. Heiland, and D. Ekeberg. 2004. Multivariate analysis of fatty acids in spores of higher basidiomycetes: A new method for chemotaxonomical classification of fungi. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci*. **800:** 303–307.
4. Clericuzio, M., F. G. Han, F. S. Pan, and O. Sterner. 1998. The sesquiterpenoid contents of fruit bodies of *Russula delica*. *Acta Chem. Scand*. **52:** 1333–1337.
5. Elmastas, M., O. Isildak, I. Turkekul, and N. Temur. 2007. Determination of antioxidant activity and antioxidant compounds in wild edible mushrooms. *J. Food Compos. Anal*. **20:** 337–345.
6. Giollan, M., J. Guillot, M. Damez, M. Dusser, P. Didier, and E. Didier. 1993. Characterization of a lectin from *Lactarius deterrimus* (research on the possible involvement of the fungal lectin in recognition between mushroom and spruce during the early stages of mycorrhizae formation). *Plant Physiol*. **101:** 513–522.
7. Girlanda, M., M. A. Selosse, D. Cafasso F. Brilli, S. Delfine, R. Fabbian, *et al*. 2006. Inefficient photosynthesis in the Mediterranean orchid *Limodorum abortivum* is mirrored by specific association to ectomycorrhizal Russulaceae. *Mol. Ecol*. **15:** 491–504.
8. Green, P. G. 1994. The ribonucleases of higher-plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol*. **45:** 421–445.
9. Guan, G. P., H. X. Wang, and T. B. Ng. 2007. A novel ribonuclease with antiproliferative activity from fresh fruiting bodies of the edible mushroom *Hypsizigus marmoreus*. *Biochim. Biophys. Acta* **1770:** 1593–1597.
10. Guillot, J., M. Giollant, M. Damez, and M. Dusser. 1991. Isolation and characterization of a lectin from the mushroom, *Lactarius deliciosus*. *J. Biochem*. **109:** 840–845.
11. Hofsteenge, J., R. Matthies, and S. R. Stone. 1989. Primary structure of a ribonuclease from porcine liver, a new member of the ribonuclease superfamily. *Biochemistry* **28:** 9806–9813.
12. Hon, C. C., T. Y. Lam, and Z. L. Shi. 2008. Evidence of the recombinant origin of a bat severe acute respiratory syndrome

(SARS)-like coronavirus and its implications on the direct ancestor of SARS coronavirus. *J. Virol*. **82:** 1819–1826.
13. Iwama, M., A. Sanda, K. Ohgi, J. Hofsteenge, and M. Irie. 1993. Purification and primary structure of a porcine kidney nonsecretory ribonuclease. *Biosci. Biotechnol. Biochem*. **57:** 2133–2138.
14. Laemmli, U. K. and M. Favre. 1973. Maturation of the head of bacteriophage T4. *J. Mol. Biol*. **80:** 575–599.
15. Lam, S. K. and T. B. Ng. 2001. Isolation of a novel thermolabile heterodimeric ribonuclease with antifungal and antiproliferative activities from roots of the sanchi ginseng *Panax notoginseng*. *Biochem. Biophys. Res. Commun.* **285:** 419–423.
16. Matousek, J., J. Soucek, J. Riha, T. R. Zankel, and S. A. Benner. 1995. Immunosuppressive activity of angiogenin in comparison with bovine seminal ribonuclease and pancreatic ribonuclease. *Comp. Biochem. Physiol. B Biochem. Mol. Biol*. **112:** 235–241.
17. Mekkriengkrai, D., T. Sando, K. Hirooka, J Sakdapipanich , Y. Tanaka, E. Fukusaki, and A. Kobayashi. 2004. Cloning and characterization of farnesyl diphosphate synthase from the rubber-producing mushroom *Lactarius chrysorrheus*. *Biosci. Biotechnol. Biochem.* **68:** 2360–2368.
18. Mock, J. W. Y., T. B. Ng, R. N. S. Wong, Q. Z. Yao, H. W. Yeung, and W. P. Fong. 1996. Demonstration of ribonuclease activity in the plant ribosome-inactivating proteins alpha- and beta-momorcharins. *Life Sci*. **59:** 1853–1859.
19. Mossakowska, D. E., K. Nyberg, and A. R. Fersht. 1989. Kinetic characterization of the recombinant ribonuclease from *Bacillus amyloliquefaciens* (Barnase) and investigation of key residues in catalysis by site-directed mutagenesis. *Biochemistry* **28:** 3843–3850.
20. Ngai, P. H. K., H. X. Wang, and T. B. Ng. 2003. Purification and characterization of a ubiquitin-like peptide with macrophage stimulating, antiproliferative and ribonuclease activities from the mushroom *Agrocybe cylindracea*. *Peptides* **24:** 639–645.
21. Ngai, P. H. K. and T. B. Ng. 2004. A ribonuclease with antimicrobial, antimitogenic and antiproliferative activities from the edible mushroom *Pleurotus sajor-caju*. *Peptides* **25:** 11–17.
22. Ng, T. B. and H. X. Wang. 2004. Flammin and velin: New ribosome inactivating polypeptides from the mushroom *Flammulina velutipes*. *Peptides* **25:** 929–933.
23. Ng, T. B. and H. X. Wang. 2004. A novel ribonuclease from fruiting bodies of the common edible mushroom *Pleurotus eryngii*. *Peptides* **25:** 1365–1368.
24. Panchak, L. V. and V. O. Antoniuk. 2007. Purification of lectin from fruiting bodies of *Lactarius rufus* (Scop.: Fr.)Fr. and its carbohydrate specificity. *Ukr. Biokhim. Zh*. **79:** 123–128.
25. Sasso, M. P., A. Carsana, E. Confalone, C. Cosi, S. Sorrentino, M. Viola, M. Palmieri, E. Russo, and A. Furia. 1991. Molecular-cloning of the gene encoding the bovine brain ribonuclease and its expression in different regions of the brain. *Nucleic Acids Res*. **19:** 6469–6474.
26. Sychrová, H., M. Tichá, and J. Kocourek. 1985. Studies on lectins. LIX. Isolation and properties of lectins from fruiting bodies of *Xerocomus chrysenteron* and *Lactarius lignyotus*. *Can. J. Biochem. Cell Biol*. **63:** 700–704.
27. Wang, H. X., S. S. Lam, 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*. **235:** 135–142.
28. Wang, H. X. and T. B. Ng. 1999. Isolation of a new ribonuclease from fresh fruiting bodies of the straw mushroom. *Biochem. Biophys. Res. Commun*. **264:** 714–718.
29. Wang, H. X. and T. B. Ng. 2000. 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.
30. Wang, H. X. and T. B. Ng. 2000. Quinqueginsin, a novel protein with anti-human immunodeficiency virus, antifungal, ribonuclease and cell-free translation-inhibitory activities from American ginseng roots. *Biochem. Biophys. Res. Commun*. **269:** 203–208.
31. Wang, H. X. and T. B. Ng. 2001. 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.
32. Wang, H. X. and T. B. Ng. 2004. A new ribonuclease from the black oyster mushroom *Pleurotus ostreatus*. *Peptides* **25:** 685–687.
33. Wang, H. X. and T. B. Ng. 2003. A novel ribonuclease from the veiled lady mushroom *Dictyophora indusiata*. *Biochem. Cell Biol.* **81:** 373–377.
34. Wang, H. X. and T. B. Ng. 2003. A ribonuclease with distinctive features from the wild green-headed mushroom *Russulus virescens*. *Biochem. Biophys. Res. Commun*. **312:** 965–968.
35. Wang, H. X. and T. B. Ng. 2004. Isolation of a new ribonuclease from fruiting bodies of the silver plate mushroom *Clitocybe maxima*. *Peptides* **25:** 935–939.
36. Wang, H. X. and T. B. Ng. 2004. Purification of a novel ribonuclease from dried fruiting bodies of the edible wild mushroom *Thelephora ganbajun*. *Biochem. Biophys. Res. Commun*. **324:** 855–859.
37. Wang, H. X. and T. B. Ng. 2006. A novel ribonuclease from fresh fruiting bodies of the portabella mushroom *Agaricus bisporus*. *Biochem. Cell Biol.* **84:** 178–183.
38. Wang, J. B., H. X. Wang, and T. B. Ng. 2007. A peptide with HIV-1 reverse transcriptase inhibitory activity from the medicinal mushroom *Russula paludosa*. *Peptides* **28:** 560–565.
39. Wong, J. H. and T. B. Ng. 2005. Lunatusin, a trypsin-stable antimicrobial peptide from lima beans (*Phaseolus lunatus* L.). *Peptides* **26:** 2086–2092.
40. Ye, X. Y. and T. B. Ng. 2002. A novel and potent ribonuclease from fruiting bodies of the mushroom *Pleurotus pulmonarius*. *Biochem. Biophys. Res. Commun.* **293:** 857–861.
41. Zheng, S. Y., C. X. Li, T. B. Ng, and H. X. Wang. 2007. A lectin with mitogenic activity from the edible wild mushroom *Boletus edulis*. *Process Biochem*. **42:** 1620–1624.