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A Novel Ribonuclease with Potent HIV-1 Reverse Transcriptase Inhibitory Activity from Cultured Mushroom *Schizophyllum commune*

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A 20-kDa ribonuclease (RNase) was purified from fresh fruiting bodies of cultured *Schizophyllum commune* mushrooms. The RNase was not adsorbed on Affi-gel blue gel but adsorbed on DEAE-cellulose and CM-cellulose. It exhibited maximal RNase activity at pH 6.0 and 70°C. It demonstrated the highest ribonucleolytic activity toward poly (U) (379.5 μ/mg), the second highest activity toward poly (C) (244.7 μ/mg), less activity toward poly (A) (167.4 μ/mg), and much weaker activity toward poly (G) (114.5 μ/mg). The RNase inhibited HIV-1 reverse transcriptase with an IC_{50} of 65 μM. No effect on [^{3}H-methyl]-thymidine uptake by lymphoma MBL2 cells and leukemia L1210 cells was observed at 100 μM concentration of the RNase. A comparison of RNases from *S. commune* and *Volvariella volvacea* revealed that they demonstrated some similarities in N-terminal amino acid sequence, optimum pH and polyhomoribonucleotide specificity. However, some differences in chromatographic behavior and molecular mass were observed.

***Keywords*:** *Schizophyllum commune,* mushroom, purification, ribonuclease

Ribonucleases are enzymes that are produced by microbes and many organisms in the animal, plant, and microbial kingdoms (Kobayashi *et al.*, 1992; Green, 1994; Watanabe *et al.*, 1995; Wang and Ng, 2001; Wang and Ng, 2003a; Fang and Ng, 2011). In mammals, ribonucleases are found in various organs such as the brain (Sasso *et al.*, 1991), liver (Hofsteenge *et al.*, 1988), kidney (Irie *et al.*, 1988), pancreas (Matousek *et al.*, 1995), and in secretions including milk (Shapiro and Vallee, 1987) and semen (Adinolfi *et al.*, 1995). A variety of biological activities have been found in ribonucleases, encompassing antifungal (Wang and Ng, 2000, 2003b; Lam and Ng, 2001), antibacterial (Ngai and Ng, 2004), HIV-1 reverse transcriptase inhibitory (Wang and Ng, 2000), angiogenic (Shapiro and Vallee, 1987), immunosuppressive and anti-mitogenic (Matousek *et al.*, 1995), and antiproliferative (Ngai and Ng, 2004) activities. Ribonucleases isolated from mushrooms may manifest different biochemical characteristics including N-terminal sequences, optimum pH, optimum temperature, and polyhomoribonucleotide specificity (Ng, 2004).

Schizophyllum commune, which is also called split gill mushroom and "white ginseng" by local people in Yunnan province of China, is a very common, wild mushroom in the genus *Schizophyllum*. It was said to be an inedible mushroom species, however, it is used as food and medicine in Yunnan, China. *S. commune* is also a model species for Microbial Genetics, and its genome has been sequenced (Ohm *et al.*, 2010). Moreover, several proteins have been purified from *S. commune*, including a cholesterol oxidase (Fukuyama and Miyake, 1979), a β-glucosidase (Desrochers *et al.*, 1981), a squalene synthase inhibitor (Kogen *et al.*, 1996), a 50-aldehyde-forming enzyme (Chen and McCormick, 1997), a cellobiose dehydrogenase (Fang *et al.*, 1998), a trehalose phosphorylase (Eis and Nidetzky, 1999), hydrophobins (Scholtmeijer *et al.*, 2001), a lectin (Han *et al.*, 2005), and a hemolysin (Han *et al.*, 2010). The aim of this study was to isolate a ribonuclease from cultured *S. commune*, and compare its characteristics with previously reported ribonucleases.

Materials and Methods

Purification scheme

S. commune strain 0805 was cultivated using the method of Han *et al.* (2010). Fresh fruiting bodies (0.65 kg) of *S. commune* were extracted with distilled water (3.5 ml/g) using a Waring blender (JYL-350A, Joyoung, China). Tris-HCl buffer (pH 7.2, 1.0 M) was added to the supernatant obtained by centrifugation of the homogenate, until the concentration of Tris reached 10 mM. The supernatant was subjected to ion exchange chromatography on a 5×20 cm column of DEAE-cellulose (Sigma, USA) in 10 mM Tris-HCl buffer (pH 7.2). After elution of unadsorbed proteins (fraction D1) with the same buffer, adsorbed proteins were desorbed sequentially with 0.2 M NaCl and 1 M NaCl in the Tris-HCl buffer to form fractions D2 and D3, respectively. Fraction D2 was dialyzed and chromatographed on a 5×15 cm of Affi-gel blue gel column (Bio-Rad, USA) in 10 mM Tris-HCl buffer (pH 7.2). Unadsorbed proteins were eluted as fraction B1. Adsorbed proteins were eluted with 1 M NaCl in the Tris-HCl buffer (pH 7.2) and collected as fraction B2. Fraction B1 was dialyzed against 10 mM NH_4OAc buffer (pH 5.0) subjected to ion exchange chromatography on a 2.5×20 cm column of CM-cellulose (Sigma, USA) in 10 mM NH_4OAc buffer (pH 5.0). After removal

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of unadsorbed proteins (fraction CM1), adsorbed proteins were eluted with 10 mM NH_4OAc buffer (pH 5.0) containing a linear gradient of 0 to 1 M NaCl and collected as fractions CM2 and CM3. Fraction CM2 was dialyzed, lyophilized and further purified on a Superdex 75 HR 10/30 column (GE Healthcare, USA) in 0.2 M NH_4HCO_3 buffer (pH 8.5) using an AKTA Purifier (GE Healthcare). The second eluted fraction (SU2) represented purified RNase.

Molecular mass determination by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and by FPLC-gel filtration

SDS-PAGE was carried out in accordance with the procedure of Laemmli and Favre (1973), using a 12% resolving gel and a 5% stacking gel. At the end of electrophoresis, the gel was stained with Coomassie brilliant blue (Sigma). FPLC-gel filtration was carried out using a Superdex 75 HR 10/30 column that had been calibrated with molecular-mass standards using an AKTA Purifier (GE Healthcare) for molecular-mass calculation.

Analysis of N-terminal amino acid sequence

Amino acid sequence analysis was carried out using an HP G1000A Edman degradation unit and an HP1000 HPLC system (Lam *et al.*, 1998).

Assay for activity of ribonuclease

The activity of the purified RNase toward yeast tRNA (Sigma) was assayed by determining the generation of acid-soluble, UV-absorbing species with the method of Wang and Ng (2003a). In brief, the RNase was incubated with 200 μg tRNA in 150 μl of MES buffer (100 mM, pH 6) at 37°C for 1 h. The reaction was terminated by introduction of 350 μl of ice-cold 3.4% perchloric acid. After leaving on ice for 15 min, the sample was centrifuged (15,000×g, 15 min) at 4°C. The Absorption at 260 nm ($A_{260\ nm}$) of the supernatant was read after appropriate dilution. One unit of enzymatic activity is defined as the amount of enzyme that brings about an increase in $A_{260\ nm}$ of one per minute in the acid-soluble fraction per ml of reaction mixture under the specified condition.

Activity of RNase toward polyhomoribonucleotides

The ribonucleolytic activity of the purified RNase toward polyhomoribonucleotides was determined with a modification of the method of Zhang *et al*. (2010b). Incubation of RNase with 100 μg poly (A), poly (C), poly (G) or poly (U) in 250 μl of 100 mM sodium acetate buffer (pH 5.0) was carried out at 37°C for 1 h, prior to addition of 250 μl of ice-cold 1.2 N 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 for poly (A), poly (G), and poly (U), or at 280 nm for poly (C).

Assay of antiproliferative activity on tumor cell lines

The assay for antiproliferative activity was conducted because this activity has been reported for many mushroom proteins (Wang *et al.*, 1995; Zhang *et al.*, 2010a) and sanchi ginseng (*Panax notoginseng*) RNase (Lam and Ng, 2001). Lymphoma MBL2 cells and leukemia L1210 cells were cultured in Dulbecco's modified Eagle's 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 supplied with 5% 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 *S. commune* ribonuclease in 100 μl DMEM medium were then added to the wells and incubated for 72 h (Zhao *et al.*, 2010). After that, [3-[4,5-dimethythiazol-2-yl]-2,5-diphenyltetrazolium bromide] (MTT) quantification assays were carried out to measure the cells' viability. Briefly, 20 μl of a 5 mg/ml solution of MTT in phosphate buffered saline was added to 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 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 RNase as negative control, sanchi ginseng RNase was used as positive control (Lam and Ng, 2001).

Assay for HIV-1 reverse transcriptase inhibitory activity

The assay for HIV-1 reverse transcriptase inhibitory activity was conducted because this activity has been reported for sanchi ginseng RNase (Lam and Ng, 2001). Instructions supplied with the assay kit from Boehringer Mannheim (Germany) were followed. The assay takes advantage of the ability of reverse transcriptase to synthesize DNA, beginning from the template/primer hybrid poly (A) oligo $(dT)_{15}$. The digoxigenin- and biotin-labeled nucleotides in an optimized ratio are incorporated into the DNA molecules 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. Biotin-labeled DNA binds to the surface of microtiter plate modules that have been precoated with streptavidin. In the next step, an antibody to digoxigenin, conjugated to peroxidase, binds to the digoxigenin-labeled DNA. In the final step, the peroxidase substrate is added. The peroxidase enzymes catalyze cleavage of the substrate, producing a colored reaction product. The absorbance of the samples at 405 nm can be measured using a microtiter plate (ELISA) reader and is directly correlated to the level of RT activity. A fixed amount (4-6 ng) of recombinant HIV-1 reverse transcriptase was used. The inhibitory activity of the isolated ribonuclease was calculated as percent inhibition as compared to a control without the *S. commune* ribonuclease (Lam and Ng, 2001; Wang and Ng, 2001; Ye and Ng, 2002b).

Assay of antifungal activity

The assay was conducted because this activity has been reported for sanchi ginseng RNase (Lam and Ng, 2001). The assay for antifungal activity toward *Mycosphaerella arachidicola* and *Physalospora piricola* was carried out in 100×15 mm petri dishes 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 portabella mushroom RNase was added to a disk. The plates were incubated at 23°C for 72 h until mycelial growth had enveloped the disks containing the control and had formed crescents of inhibition around disks containing samples with antifungal activity. Sanchi ginseng RNase was used as positive control (Lam and Ng, 2001).

Results

Ion exchange chromatography of the fruiting body extracted on DEAE-cellulose yielded three fractions D1, D2, and D3. RNase activity was found mainly in the adsorbed fraction D2 (Table 1). D2 was separated into one unadsorbed (larger)

Table 1. Yields and RNase activities of chromatographic fractions at various purification stages (from 650 g fresh fruiting bodies, 0.1 M Mes buffer, pH 6.0, 37°C for 15 min)

	Yield (mg)	RNase activity (U/mg)		Yield (mg)	RNase activity (U/mg)
Extract	1395	72.8	CM1	62.0	< 1
D1	298	< 1	CM2	46.1	1050.6
D2	411	189.3	CM3	50.8	56.8
D3	318	19.6	SU1	10.8	70.1
B1	229	281.9	SU2	13.4	2892.7
B2	80.6	< 1	SU3	8.5	148.5

fraction (B1) and one adsorbed fraction (B2). Fraction B1 was the only fraction with RNase activity (Table 1). B1 was resolved on CM-cellulose into an unadsorbed fraction CM1 in which no RNase activity was detected, two adsorbed fractions CM2 and CM3 (Fig. 1). RNase activity was concentrated in fraction CM2 (Table 1). Fraction CM2 was resolved into three fractions, SU1, SU2, and SU3 upon gel filtration on Superdex 75 (Fig. 2). RNase activity resided in the second (also largest) fraction SU2 (Table 1). Protein in fraction SU2 appeared as a single band with a molecular mass of 20 kDa (Fig. 3). Other than slight similarity to *Volvariella volvacea*

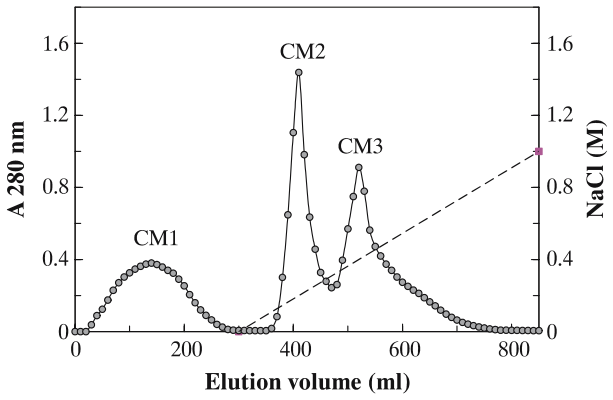

Fig. 1. Ion exchange chromatography on CM-cellulose. Sample: fraction D2 which was adsorbed on DEAE-cellulose. Column dimensions: 2.5×20 cm. Starting buffer: 10 mM NH_4OAc (pH 5). Slanting dotted line across the right side of the chromatogram represents linear 0-1 M NaCl gradient used to elute adsorbed proteins.

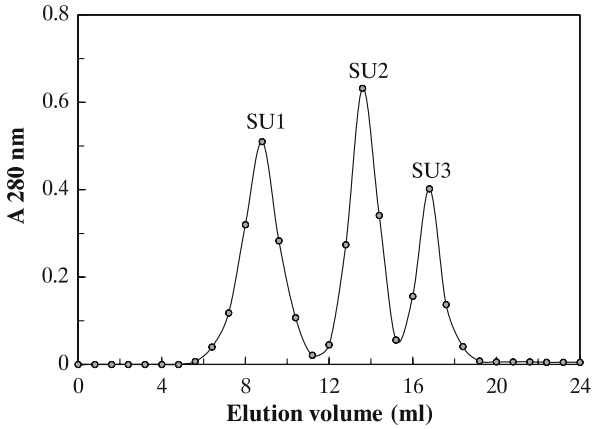

Fig. 2. FPLC-gel filtration of fraction CM2 on a Superdex 75 HR10/30 column. Buffer: 0.2 M NH_4HCO_3 (pH 8.5). Flow rate: 0.4 ml/min. Fraction size: 0.8 ml.

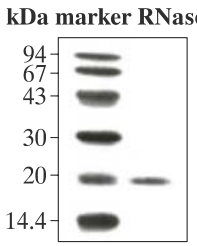

Fig. 3. SDS-PAGE of purified *S. commune* RNase (fraction SU2). The molecular mass of the marker proteins are, from top downward, 94, 67, 43, 30, 20, and 14.4 kDa.

Table 2. N-terminal sequence of *S. commune* ribonuclease in comparison with other mushroom ribonucleases

RNase		N-terminal sequence
SC	:	TPYLDYLAAL QADGPVVPFIRNWEGALSIS
VV	:	APYV--QLFRPLIQPQVLATFAIANNMAQY
AB	:	ACAANNAARRYYASSVGNNN
CM	:	ETAHTHAGIQYSTVDVNNSIMKAVGGGAGN
DI	:	GQPRQPQPQLLV
GL	:	HLPBVPSFAYGSIKVYIN
IL	:	VNSGCGTSGAESCSNSDDGTCCFEAPGGLL
LE	:	ISSGCGTTGALSCSSNAKGTCCFEAPGGLI
PE	:	GEVVQYYP
PO	:	ETGVRSCNCAGRSFTGTDVTNAIRSARAGGSGN
PP	:	AISANNERKGVNQQSVQNTYQENDV
PS	:	DNGEAGRAAR
PT	:	ALTAQDNRVRVGNRIVGNNFNFAAVQAAYY
RV	:	TDHTLDTMMTHTLRD
TG	:	DADIAVWAPPVNAQN

Identical amino acid residues are underscored. SC, *Schizophyllum commune* (this study); AB, *Agaricus bisporus* (Wang and Ng, 2006); CM, *Clitocybe maxima* (Wang and Ng, 2004d); DI, *Dictyophora indusiata* (Wang and Ng, 2003a); GL, *Ganoderma lucidum* (Wang *et al.*, 2004); IL, *Irpex lacteus* (Watanabe *et al.*, 1995); LE, *Lentinus edodes* (Kobayashi *et al.*, 1992); PE, *Pleurotus eryngii* (Wang and Ng, 2004b); PO, *Pleurotus ostreatus* (Wang and Ng, 2004c); PP, *Pleurotus pulmonarius* (Ye and Ng, 2002); PS, *Pleurotus sajor-caju* (Ngai and Ng, 2004); PT, *Pleurotus tuber-regium* (Wang *et al.*, 1998); RV, *Russulus virescens* (Wang and Ng, 2003a); TG, *Thelephora ganbajun* (Wang and Ng, 2004e); and VV, *Volvariella volvacea* (Wang and Ng, 1999).

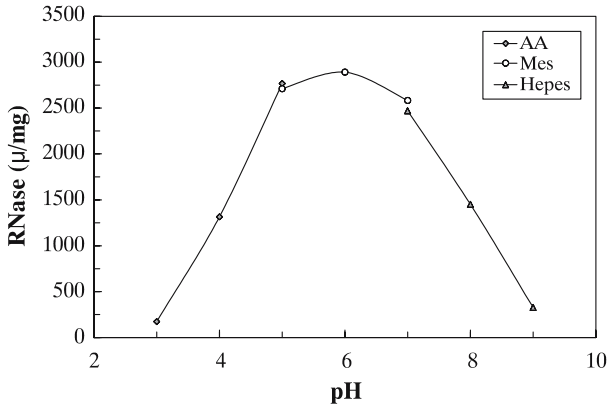

Fig. 4. pH dependence of *S. commune* RNase. Temperature used: 37°C. Duration of incubation: 15 min. Buffer concentration: 0.1 M.

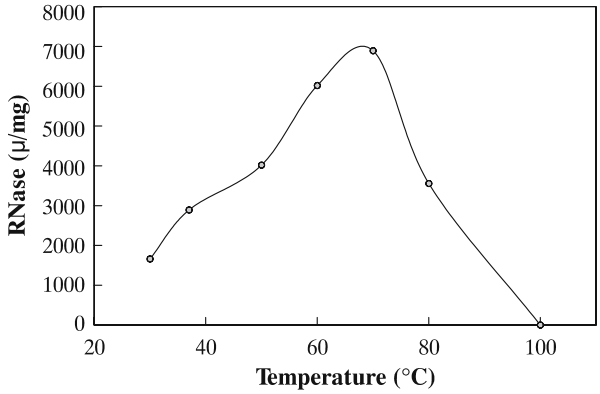

Fig. 5. Temperature dependence of *S. commune* RNase. Buffer used: pH 4.5 0.1 M NH_4OAc buffer. 37°C for 15 min.

RNase (Wang and Ng, 1999), its N-terminal sequence appeared largely different from those of previously reported mushroom RNases (Table 2). The optimum pH (Fig. 4) and temperature (Fig. 5) for the purified RNase were pH 6.0 and 70°C,

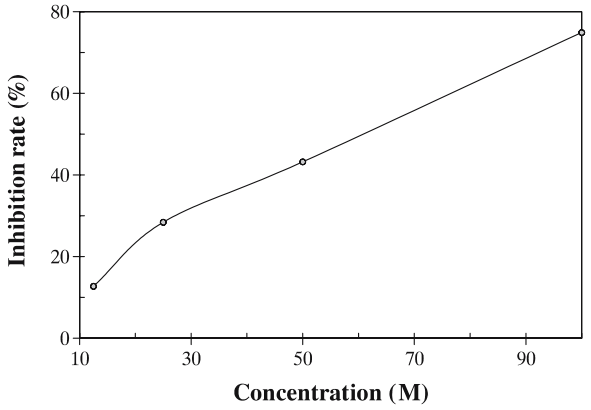

Fig. 6. Inhibitory effect of *S. commune* RNase on activity of HIV-1 RT. The IC_{50} is 65 μM.

respectively. The RNase exerted a ribonucleolytic activity of 379.5, 244.7, 167.4, and 114.5 μ/mg for poly (U), poly (C), poly (A), and poly (G), respectively. It inhibited HIV-1 reverse transcriptase with an IC_{50} of 65 μM (Fig. 6). No effect on MTT uptake by lymphoma MBL2 cells and leukemia L1210 cells was observed at 100 μM RNase (data not shown). No antifungal activity was detected for the isolated RNase (data not shown). Table 3 shows a comparison of the characteristics of RNases from *S. commune* and *V. volvacea*. It can be seen that there are similarities in the optimum pH and polyhomoribonucleotide specificity. Some differences in chromatographic behavior and molecular mass exist.

Discussion

The ribonuclease isolated from the split gill mushroom *S. commune* in the present study displayed an N-terminal sequence distinct from those of published mushroom ribonucleases indicating that it is a novel ribonuclease. It showed only slight homology to straw mushroom (*V. volvacea*) RNase. Its molecular size appeared within the range exhibited by mushroom ribonucleases, being smaller than that of its straw

Table 3. Comparison of characteristics of RNases from *S. commune* and *V. volvacea*

Characteristics	*S. commune* RNase	*V. volvacea* RNase
Molecular mass (kDa)	20	42.5
Chromatographic behavior		
DEAE-Cellulose	adsorbed	unadsorbed
Affi-gel blue gel	unadsorbed	adsorbed
CM ion exchange gel	adsorbed	adsorbed
Optimum temperature	70°C	Not determined
Optimum pH	6	6.5-7.5
Poly homoribonucleotide specificity (relative activity)	Poly U (100%)	Poly U (100%)
	Poly C (65%)	Poly C (10%)
	Poly A (43%)	Poly A (negligible)
	Poly G (30%)	Poly G (negligible)
Antifungal activity	No	Not determined
Antiproliferative activity	No	Not determined
HIV-1 RT inhibitort activity	IC_{50}=65 μM	Not determined

mushroom counterpart (42 kDa) (Wang and Ng, 1999), but larger than that of oyster mushroom RNase (11 kDa) (Nomura *et al.*, 1994; Ye and Ng, 2002a). Its ribonucleolytic activity toward four polyhomoribonucleotides had a unique specificity. It was high toward poly (A) and poly (C) and it had approximately one-quarter as much the activity toward poly (U) and poly (G). The optimum pH was 6 and optimum temperature was 70°C for *S. commune* ribonuclease. Its optimum pH was the same as that of *Pleurotus pulmonarius* ribonuclease (Ye and Ng, 2002), but very different from the optimum pH of 4.5 for *Russulus virescens* ribonuclease (Wang and Ng, 2003a) and 4.0 for *Ganoderma lucidum* ribonuclease (Wang *et al.*, 2004). The temperature dependence curve for the activity of *S. commune* ribonuclease indicated that it was a fairly thermostable enzyme. It retained more than half of its maximal activity at 80°C and is totally inactivated only at 100°C.

Its lack of antiproliferative activity toward tumor cell lines is in contrast to the inhibitory action of *P. sajor-caju* ribonuclease (Ngai and Ng, 2004), and to brown oyster mushroom ribonuclease (Xia *et al.*, 2005) on [methyl-^{3}H] thymidine uptake by tumor cells. Some ribonucleases e.g. those from sanchi ginseng (Lam and Ng, 2001), American ginseng (Wang and Ng, 2000), and Chinese ginseng (Wang and Ng, 2004a) exhibited antifungal activity. In contrast, *S. commune* RNase and other mushroom RNases reported to date lack this activity (Wang and Ng, 2004b; Guan *et al.*, 2007; Zhang *et al.*, 2010b). The inhibitory potency toward HIV-1 reverse transcriptase was reminiscent of similar activities reported for its counterparts in other mushroom species. Its potency ranks relatively high among the anti-HIV natural products (Ng *et al.*, 1997). The mechanism is probably protein-protein interaction, like the inhibition of HIV-1 reverse transcriptase by HIV-1 protease (Bottcher and Grosse, 1997). It deserves attention that not all mushroom ribonucleases exhibit HIV-1 reverse transcriptase inhibitory activity, e.g. *P. ostreatus* RNase (Wang and Ng, 2004c; Xia *et al.*, 2005).

In summary, *S. commune* has a mushroom ribonuclease with some unique features. Unlike other mushroom RNases, *S. commune* RNase is not adsorbed on Affi-gel blue gel, it lacks antiproliferative activity toward tumor cells, and has a distinctive polyhomoribonucleotide specificity. It is somewhat similar to *V. volvacea* RNase in N-terminal sequence, optimum pH and polyhomoribonucleotide specificity, both belonging to order *Agaricalas*. Nevertheless, their chromatographic behavior and molecular mass differ. The isolation of ribonuclease from *S. commune* in this report adds to the literature regarding this mushroom.

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