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Purification and characterization of a ribonuclease from the wild edible mushroom *Armillaria luteo-virens*

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A 15 kDa ribonuclease (RNase) was purified from dried fruiting bodies of the wild edible mushroom *Armillaria luteo-virens*. The simple 4-step purification protocol involved ion-exchange chromatography on DEAE-cellulose, affinity chromatography on Affi-gel blue gel, ion-exchange chromatography on SP-Sepharose and a final gel filtration by FPLC on Superdex-75. The RNase was unadsorbed on Affi-gel blue gel, but adsorbed on DEAE-cellulose and SP-Sepharose. The N-terminal amino acid sequence of purified RNase was AGVQYKLTILLV, which showed low sequence homology to those of previously reported RNases. The optimal pH and temperature of the enzyme were very close to 4.0 and 70°C, respectively. The enzyme showed considerably high ribonucleolytic activity and broad specificity towards polyhomoribonucleotides, with a specificity of poly(U)>poly(C)>poly (G)>poly(A). The ribonucleolytic activities towards poly(U), poly(C), poly(G) and poly(A) were 279.5, 184.1, 69.9 and 52.3 U/mg, respectively.

Keywords: *Armillaria luteo-virens*, Muchroom, Purification, Ribonuclease

Ribonucleases (RNases), which can degrade RNA into smaller components, have been isolated and characterized from various organisms, including animals, plants and microbes[1-3]. RNases manifest diverse functions in a variety of physiological processes, including maturation and degradation of RNA molecules, modulation of host immune response and resistance towards RNA viruses. In addition, RNases also play a critical role in angiogenesis and self-incompatibility in flowering plants[4].

In Chinese medicine, mushrooms are popular for their various medicinal functions as well as their delicious taste and nutritive value. RNases have been isolated from mycelia or fruiting bodies of a variety of mushroom species, including *Agaricus bisporus*, *Clitocybe maxima*, *Diotyophora indusiata*, *Ganoderma lucidum*, *Irpex lacteus*, *Lentinus edodes*, *Pleurotus eryngii*, *P. pulmonarius*, *Russulus virescens*, *Volvariella volvacea* etc[5-14]. They are reported to exhibit many bioactivities such as anti-proliferative, antitumor, antibacterial, antifungal, antiviral, HIV-1 reverse transcriptase inhibitory and immunosuppressive[11,15-18]. Based on the known data, RNases have been reported to manifest a wide molecular mass range, low N-terminal amino acid sequence homology, diverse temperature and pH optima and different polyhomoribonucleotide specificity. Thus, it would be worthwhile to isolate novel RNases from mushroom species that have not been examined.

Armillaria luteo-virens (Phylum Basidiomycota, Order Agaricales, Family Physalacriaceae), also called yellow mushroom by local people, is a wild edible mushroom found in Qinghai province of China. An inulin-specific lectin with mitogenic activity towards spleen cells and anti-proliferative activity towards tumor cells has been isolated from *A. luteo-virens*[19]. It is also used for the production of betulinic acid[20]. Here, we report isolation of a ribonuclease from *A. luteo-virens* and its biochemical characteristics and biological activities have been compared with other previously isolated RNases.

Materials and Methods

Purification protocol

Dried fruiting bodies (200 g) of the wild edible mushroom *Armillaria luteo-virens* were extracted with

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distilled water (2 ml/g) using a Waring blender. Tris-HCl buffer (pH 7.2, 1 M) was added to the supernatant, obtained by centrifugation (12,000 rpm, 4°C, 15 min) of the homogenate, until the final 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) using 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 and 1 M NaCl in the Tris-HCl buffer to form fractions D2 and D3, respectively (Flow rate: 2.0 ml/min, Fraction size: 10 ml).

Fraction D2 contained major RNase activity and was subsequently chromatographed on a 5 × 18 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 sequentially eluted with 1 M NaCl in the Tris-HCl buffer and collected as fractions B2 (Flow rate: 2.0 ml/min; Fraction size: 10 ml).

Fraction B1 with RNase activity was subsequently dialyzed against 10 mM sodium acetate (NaAc) buffer (pH 5.0) subjected to ion-exchange chromatography on a 2.5 × 20 cm of SP-Sepharose (GE Healthacre, Sigma) in the same NaAc buffer (pH 5.0). After removal of unadsorbed proteins (fraction S1), adsorbed proteins were eluted with a linear NaCl concentration (0-1 M) gradient in 10 mM NaAc buffer (pH 5.0) (Flow rate: 2.0 ml/min; Fraction size: 10 ml). The first adsorbed fraction (S2) was finally purified on a Superdex 75 HR 10/30 column (GE Healthcare, USA) in 0.2 M NH_4HCO_3 buffer (pH 8.5) (Flow rate: 0.4 ml/min; Fraction size: 0.8 ml).

Molecular mass determination on FPLC-gel filtration was carried out using the molecular mass standards (AKTA Purifier, GE Healthcare, USA). A standard curve of elution volume-lg*Mr* was obtained. The molecular mass of purified enzyme was calculated using the curve and its elution volume.

Electrophoretic methods

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in accordance with the procedure of Laemmli and Favre[21], using a 12% resolving gel and a 5% stacking gel. At the end of electrophoresis, the gel was stained with Coomassie brilliant blue R-250 (Sigma, USA). The molecular mass of purified enzyme was calculated from the plot of log molecular mass *versus* electrophoretic mobility of molecular weight markers (GE Healthcare, USA).

N-terminal amino acid sequence determination

Amino acid sequence analysis was carried out using an HP G1000A Edman degradation unit and an HP1000 HPLC system[18].

Enzyme assay

RNase activity was assayed using the method of Wang and Ng[8]. In brief, RNase extract (5 µl) was incubated in a reaction mixture containing 200 µg yeast tRNA substrate (Sigma, USA) and 150 µl Mes buffer (100 mM, pH 6.0) at 37°C for 15 min. Subsequently, the reaction was terminated by 345 µl of ice-cold 5.0% perchloric acid. After standing on ice for 15 min, the sample was centrifuged (12,000 rpm, 5 min) at 4°C. The OD_{260} of supernatant was read after appropriate dilution. One unit (U) of enzymatic activity was defined as the amount of enzyme that brought about an increase in OD_{260} of one per min in the acid-soluble fraction per ml of reaction mixture under the specified condition. Protein concentration was measured by the Bradford's method using bovine serum albumin (BSA) as a standard[22].

Determination of pH and temperature optima

In the assay for the optimal pH value, a series of assay buffers with different pH values were used. The assay buffers were prepared in 100 mM ammonium acetate buffer (AA, pH 3.0-5.0), 100 mM Mes buffer (pH 5.0-7.0) and 100 mM Hepes buffer (pH 7.0-9.0). The assay temperature was 37°C. To determine the optimal temperature, the reaction mixture was incubated at 30-100°C. The assay buffer was the same buffer used in the standard RNase assay above.

Ribonucleolytic activity towards polyhomoribonucleotides

The ribonucleolytic activity of purified RNase towards polyhomoribonucleotides was determined with 100 µg poly(A), poly(C), poly(G) or poly(U) (Sigma, USA) in 250 µl of 100 mM sodium acetate buffer (pH 5.0)[18]. The reaction mixture was incubated at 37°C for 1 h. Subsequently, the reaction was terminated by 250 µl of ice-cold 1.2 M perchloric acid containing 20 mM lanthanum nitrate. After leaving on ice for 15 min, the sample was centrifuged at 12,000 rpm for 5 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). Enzyme solution terminated without pre-incubation was used as control.

Results

Enzyme purification

A summary of the purification schedule is shown in Table 1. Ion-exchange chromatography of the extract on DEAE-cellulose yielded three fractions D1, D2 and D3 containing similar amounts of proteins (Fig. 1a). Major RNase activity was found in the adsorbed fraction D2. The fraction D2 was subsequently separated on Affi-gel blue gel into an unadsorbed and also the largest fraction (B1) and an adsorbed fraction (B2) (Fig. 1b).

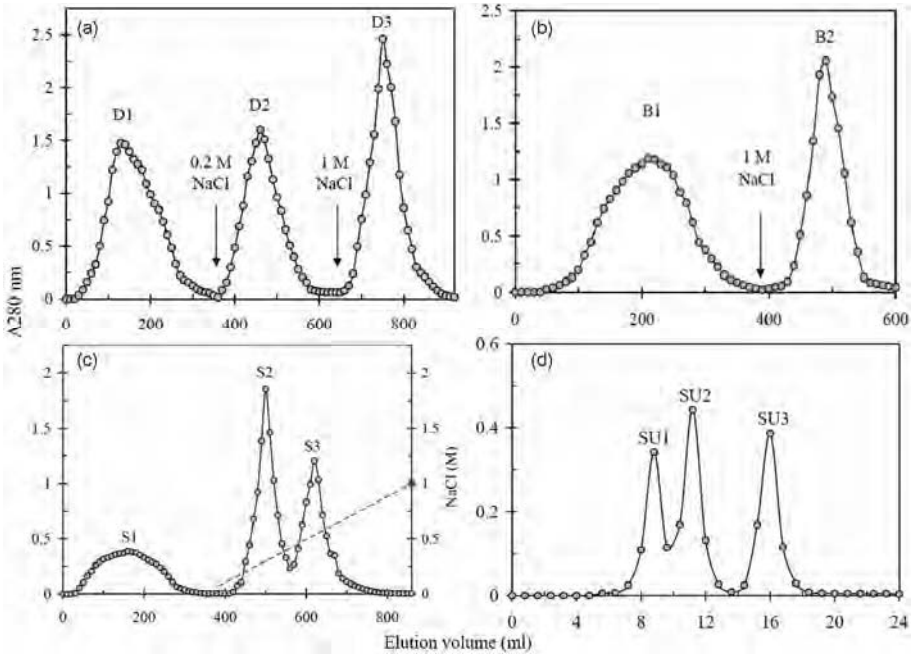

Fig. 1—Elution profiles of the purified *Armillaria luteo-virens* RNase [a): Ion-exchange chromatography on DEAE-cellulose. Sample: supernatant after final concentration. Column dimensions: 5 × 20 cm. Elution buffer: 10 mM Tris-HCl buffer (pH 7.2); (b): Affinity chromatography on Affi-gel blue gel. Sample: fraction D2 which was eluted from DEAE with 0.2 M NaCl in the Tris-HCl buffer (pH 7.2). Column dimensions: 5 × 18 cm. Elution buffer: 10 mM Tris-HCl buffer (pH 7.2); (c) Ion-exchange chromatography on SP-Sepharose. Sample: fraction B1 which was unadsorbed on Affi-gel blue gel. Column dimensions: 2.5 × 20 cm. Elution buffer: 10 mM NaAc (pH 5.0). Slanting dotted line across the right side of the chromatogram represents linear 0-1 M NaCl gradient used to elute adsorbed proteins; (d): FPLC-gel filtration on a Superdex-75 HR10/30 column. Sample: fraction S2 which was adsorbed on SP-Sepharose. Column dimensions: HR 10/30 column. Elution buffer: 0.2 M NH_4HCO_3 (pH 8.5)]

Table 1—Yield and RNase activities at each purification step (from 200 g dried fruiting body, activity assayed at 0.1 M Mes buffer, pH 6.0, 37°C/15 min)

Purification step	Total protein (mg)	RNase activity (U/mg)	Total activity (U)	Recovery of activity (%)	Purification fold
Extract	2760	60.9	168084	100	**1**
D1	693	< 2	-	-	-
D2	**545**	**174.6**	**95157**	**56.6**	**2.9**
D3	661	21.0	13881	8.3	0.3
B1	**231**	**329.1**	**76022**	**45.2**	**5.4**
B2	158	< 2	-	-	-
S1	61.6	< 2	-	-	-
S2	**48.7**	**1111.5**	**54130**	**32.2**	**18.3**
S3	41.5	118.2	4905.3	2.9	1.9
SU1	9.9	268.8	2661	1.6	4.4
SU2	**12.8**	**3165.0**	**40512**	**24.1**	**52.0**
SU3	11.2	< 2	-	-	-

Values given in bold represent RNase active fraction

Fraction B1 was the only fraction with RNase activity, which was fractionated on SP-Sepharose into an unadsorbed fraction S1 devoid of RNase activity, a large adsorbed fraction S2 with the bulk of RNase activity and a smaller adsorbed fraction S3 with slight RNase activity (Fig. 1c). Fraction S2 was then resolved into three fractions — SU1, SU2 and SU3 upon gel filtration using Superdex-75 (Fig. 1d). Fraction SU2 maintained the RNase activity and possessed a molecular mass of 15 kDa, as estimated by FPLC. On SDS-PAGE, fraction SU2 appeared as a single band with a molecular mass of 15 kDa (Fig. 2). The results of FPLC and SDS-PAGE indicated that the purified RNase was a monomeric protein with a molecular mass of 15 kDa. An overall 52.0-fold purification and activity recovery of 24.1% were achieved.

Analysis of N-terminal amino acid sequence

The N-terminal amino acid sequence of purified enzyme was determined to be AGVQYKLTILLV. An N-terminal amino acid sequence comparison of *A. luteo-virens* RNase with other mushroom RNases previously described is shown in Table 2 using Blast tool in NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and DNAMAN 6.0 software.

pH and temperature optima

The pH and temperature optima of purified RNase were determined with yeast tRNA and the results are shown in Figs 3 and 4. An optimal pH value very close to 4.0 was required for maximal activity of purified enzyme. The RNase activity increased as the pH value was raised from 3.0 to about 4.0 and with

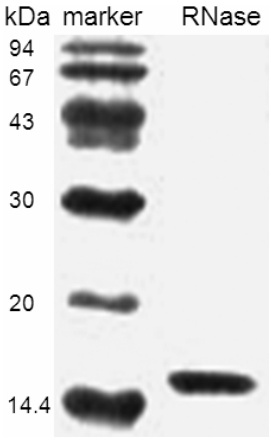

Fig. 2—SDS-PAGE of purified RNase (fraction SU2) [Lane 1: Molecular weight markers, from top downwards, phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa) and lactalbumin (14.4 kDa); and lane 2, purified RNase]

Table 2—Comparison of N-terminal amino acid sequence of *Armillaria luteo-virens* RNase and other mushroom RNases

Species	N-Terminal sequence		Ref./GenBank No.	
Armillaria luteo-virens	1	AGVQY KLTIL LV	12	This Study
Agaricus bisporus	386	H**G**Q**QY** T**L**E**I**I GS	407	BAG54998.1
Clitocybe maxima	6	**AG**I**QY** STVDV NN	17	11
Dictyophora indusiata	1	GQPRQ PQPQ**L** **LV**	12	10
Flammulina velutipes	194	**A**F**VQY** PTYKM **L**A	215	BAJ06630.1
Ganoderma lucidum	3	**AA**LGL IISA**L** **L**T	14	BAF65239.1
Grifola frondosa	244	L**G**CGF NG**T**V**L** QE	255	BAE80702.1
Hericium erinaceum	31	**AG**RS**Y** SSSNI AD	42	BAG15897.1
Irpex lacteus	399	S**G**HGT S**LT**LS I**V**	410	BAC00516.1
Lentinula edodes	269	S**G**IK**Y** P**L**KTG SP	280	BAC81429.1
Lenzites betulinus	217	S**G**I**Q**P SNKTT **Y**S**LT**Q **L**QT	234	BAG38684.1
Pholiota nameko	393	T**G**TSH TDR**Y**T **L**S**I**S T	409	BAC02943.1
Pleurotus eryngii	386	T**G**SSH SQD**Y**T **LTI**VG A	401	BAD12193.1
Pleurotus ostreatus	11	L**G**ALF SVQAA I**V**	22	BAG14392.1
Pleurotus pulmonarius	8	K**GV**NQ QSVQN TY	19	14
Pleurotus sajor-caju	1	DN**G**EA GRAAR	10	P84528.1
Pleurotus tuber-regium	15	V**G**NNF NFAAV QA	26	26
Russulus virescens	1	TDHTL DTMMT HT	12	9
Thelephora ganbajun	1	D**A**DIA VWAPP VNAQN	15	P84784.1
Volvariella volvacea	10	IQP**Q**K V**L**ATF AI	21	8

Identical amino acid residues are bold and underlined.

further increase in pH an abrupt drop in activity was observed; there was an approx. 90% decrease in activity as the pH reached 9.0 (Fig. 3).

The maximal activity of purified RNase was achieved at a temperature close to 70°C. RNase activity increased steadily when the temperature was raised from 30 to 70°C and the activity decreased rapidly at temperature higher than 80°C. The purified RNase lost more than 95% activity, when the temperature was raised to 100°C (Fig. 4).

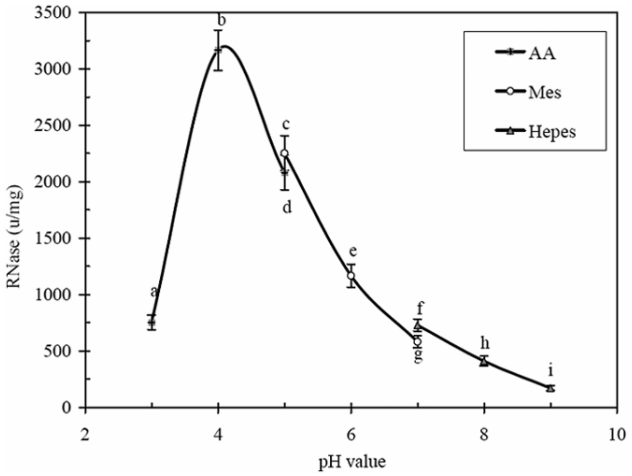

Fig. 3—pH dependence of purified RNase [Temperature used: 37°C. Duration of incubation: 15 min. Buffer concentration: 0.1 M. Each value in both panels represents the means ± SD (n = 3). Different letters (a, b, c…) next to the data points indicate statistically significant differences ($p<0.05$) when the data were analyzed by analysis of variance, followed by Duncan's multiple range test]

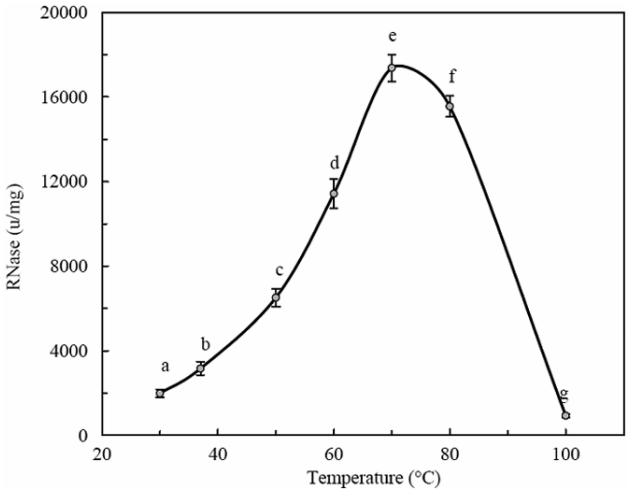

Fig. 4—Temperature dependence of the purified RNase [Buffer used: pH 4.5 0.1 M NH_4OAc buffer. 37°C for 15 min. Each value in both panels represents the means ± SD (n = 3). Different letters (a, b, c…) next to the data points indicate statistically significant differences ($p<0.05$) when the data were analyzed by analysis of variance, followed by Duncan's multiple range test]

Polyhomoribonucleotide specificity

The purified RNase demonstrated an activity of 279.5, 184.1, 69.9 and 52.3 U/mg towards poly(U), poly(C), poly(G) and poly(A), respectively.

Discussion

Based on their molecular masses, RNases have been classified into two families T1 and T2, which manifest molecular mass ranges of 11-12 kDa and 24-36 kDa, respectively[4]. Many mushroom RNases reported previously have a molecular mass range of 12-15 kDa, such as RNases from *Pleurotus sajor-caju* (12 kDa)[23], *Termitomyces globules* (13 kDa)[24], *Pleurotus pulmonarius* (14 kDa)[14], *L. shimeiji* (14.5 kDa)[25] and *Pleurotus eryngii* (16 kDa)[7]. On the other hand, mushroom RNases from *Russulus virescens* (28 kDa)[9], *Pleurotus tuber-regium* (29 kDa)[26], *Thelephora ganbajun* (30 kDa)[27] and *Volvariella volvacea* (42 kDa)[8] fall well in a molecular mass range of 30-40 kDa. It suggests that mushroom RNases can also be divided into the two families T1 and T2 based on their molecular masses. The purified RNase from *A. luteo-virens* belonged to the family T1, as it manifested molecular mass of 15 kDa, which was very close to the mass range of RNase family T1. Blast search on N-terminal amino acid sequence of purified RNase showed low sequence homology to those of previously reported RNases. From the results, it could be suggested that *A. luteo-virens* RNase might be encoded by different RNase genes.

In previous reports, a 28 kDa RNase from *Dictyophora indusiata* (veiled lady mushroom) has demonstrated a pH optimum of 4-4.5 and a temperature optimum of 60°C[10]. A RNase from mushroom *P. pulmonarius* manifests an optimal pH of 7.0 and an optimal temperature of 55°C[14]. A RNase from *R. virescens* (green-headed mushroom) has shown a pH optimum of 4.5 and a temperature optimum of 60°C[9]. However, RNases from *P. eryngii* and *P. tuber-regium* manifest a pH optimum of 6.5 and *P. ostreatus* RNase a pH optimum of 8.0[7,26,28].

The optimal pH of purified RNase from *A. luteo-virens* was very close to that of *D. indusiata* and *R. virescens*, but was lower than that of *P. eryngii*, *P. tuber-regium*, *P. pulmonarius* and *P. ostreatus*. Compared with other reported mushroom RNases, the optimal temperature of *P. ostreatus* RNase is similar to that of RNases from *Clitocybe maxima* and *L. shimeiji*, but is higher than that of RNases from *Thelephora ganbajun* (40°C),

P. pulmonarius (55°C), *D. indusiata* (60°C) and *R. virescens* (60°C)[9,10,14,27]. Enzyme activity of another RNase from *P. ostreatus* does not vary appreciably over the temperature range 30-60°C, but drops when the temperature is reduced to 20°C or raised to above 70°C[29].

A RNase from *L. shimeiji* manifests same specificity order of poly(U)>poly(C)>poly(G) >poly(A) as *A. luteo-virens* RNase, with the highest ribonucleolytic activity towards poly(U) of 14.2 U/mg[25]. *T. globulus* RNase exhibits a highest ribonucleolytic activity towards poly(C) (40.0 U/mg) and specificity order of poly(C)>poly(A)>poly(U)>poly(G)[24]. *P. pulmonarius* RNases are specific towards poly(C), poly(A) and poly(G), whereas *P. ostreatus* RNases has demonstrated a specificity towards poly(G)[14,28]. Compared with reported mushroom RNases, *A. luteo-virens* RNase manifested considerably high ribonucleolytic activity and broad specificity towards polyhomoribonucleotides.

In summary, a new RNase having the molecular mass of 15 kDa, optimum pH temperature close to pH 4.0 and 70°C respectively and with a unique N-terminal amino acid sequence of AGVQYKLTILLV was purified from the wild mushroom *A. luteo-virens* by employing liquid chromatography techniques. The enzyme showed considerably high ribonucleolytic activity and broad specificity towards polyhomoribonucleotides, with the highest ribonucleolytic activity towards poly(U) of 279.5 U/mg.

Acknowledgments

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