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Purification and Characterization of a New Ribonuclease from Fruiting Bodies of the Oyster Mushroom *Pleurotus ostreatus*

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Abstract: A ribonuclease (RNase), possessing an *N*-terminal sequence disparate from those of ribonucleases from other mushrooms and previously isolated *Pleurotus ostreatus* RNases, was purified from the fruiting bodies of the edible mushroom *Pleurotus ostreatus*. The *N*-terminal sequence of *Pleurotus ostreatus* RNase did not manifest homology even to a previously reported RNase from the same mushroom. The ribonuclease was adsorbed on CM-Sepharose and Mono S. It exhibited a molecular mass of 12 kDa in both sodium dodecyl sulphate-polyacrylamide gel electrophoresis and gel filtration on Superdex 75. The ribonuclease displayed an activity of 11 490 U/mg on yeast tRNA. The highest ribonuclease activity was exhibited toward poly U, followed by poly A and poly C. No activity was shown toward poly G. The optimal pH for its activity was 7 and the optimal temperature was 55 °C. It inhibited cell-free translation in a rabbit reticulocyte lysate with an IC_{50} of 240 nM. Copyright © 2003 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: ribonuclease; mushroom; *Pleurotus ostreatus*; fruiting body

INTRODUCTION

Ribonucleases (RNases) from a diversity of organisms in the animal and plant kingdoms have been studied. RNases have been isolated from vertebrate tissues including the brain [1], pancreas [2], liver [3], kidney [4] and ovary [5]. Milk [6] and seminal [7] RNases, as well as RNases from plants have been characterized.

RNases have been purified from mushrooms including *Irpex lacteus* [9], *Lentinus edodes* [10], *Pleurotus ostreatus* [11], *Pleurotus tuber-regium* [12] and *Volvariella volvacea* [13]. Only RNases from *I. lacteus* [9] and *Lentinus edodes* [10] demonstrate sequence homology [9,10]. Another RNase with a lower molecular weight than the previously reported RNase has been detected in *Pleurotus ostreatus* [14]. The results of the present investigation revealed a *Pleurotus ostreatus* RNase with characteristics different from the previously reported RNases from the same species [14]. The RNase reported in this paper was obtained with a lower yield but a higher specific activity compared with the RNase we prepared on an earlier occasion [14].

MATERIALS AND METHODS

Isolation Procedure

Fresh mushrooms (*Pleurotus ostreatus*) were purchased from a local supplier. The mushrooms were homogenized in 20 mM NH_4OAc buffer (pH 4.6). After centrifugation the supernatant obtained was applied on a column of CM-Sepharose (2.5 × 18 cm) and eluted with the same buffer. Following removal of unadsorbed material, the adsorbed protein was desorbed with a linear gradient of 0–0.5 M NaCl in 20 mM NH_4OAc buffer (pH 4.6). The adsorbed protein

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was dialysed and then chromatographed on a column of Mono S (Pharmacia) equilibrated and eluted with 20 mM NH_4OAc buffer (pH 4.6). Unadsorbed protein was eluted with the same buffer. Adsorbed protein was eluted with a linear gradient of 0–0.3 M NaCl in the same buffer. The active adsorbed peak from the Mono S column was further purified by FPLC — gel filtration on Superdex 75 (Pharmacia) in 0.2 M NH_4HCO_3 buffer (pH 8.6).

Molecular Mass Determination by Sodium Dodecyl Sulphate-polyacrylamide Gel Electrophoresis (SDS-PAGE) and by FPLC-gel Filtration

SDS-PAGE was carried out in accordance with the procedure of Laemmli and Favre [14]. At the end of electrophoresis, the gel was stained with Coomassie brilliant blue. FPLC-gel filtration was carried out using a Superdex 75 column which had been calibrated with molecular mass standards (Amersham Pharmacia Biotech).

Analysis of *N*-terminal Amino acid Sequence

Amino acid sequence analysis was carried out using an HP Gl000A Edman degradation unit and an HP1000 HPLC system [15].

Activity of *P. ostreatus* Ribonuclease toward Yeast tRNA

The activity of *P. ostreatus* RNase toward tRNA was assayed by determining the generation of acid-soluble, UV-absorbing species with the method of Lam *et al.* [16]. The RNase was incubated with 200 μg of yeast tRNA (Sigma) in Tris-HCl buffer (pH 7.0) at 37 °C for 15 min. The reaction was terminated by introduction of perchloric acid. After leaving on ice, the sample was centrifuged. The OD_{260} of the supernatant was read. One unit of enzymatic activity is defined as the amount of enzyme that brings about an increase in OD_{260} of 1 per min in the acid-soluble fraction per ml of reaction mixture under the specified condition.

Activity of *P. ostreatus* RNase toward Polyhomoribonucleotides

Incubation of *P. ostreatus* RNase with 200 μg of polyA, polyC, polyG or polyU in 250 μl of 100 mM Tris-HCl buffer (pH 7.0) was carried out at 37 °C for 1 h, prior to addition of perchloric acid containing lanthanum nitrate. After standing on ice, the sample was centrifuged. The absorbance of the supernatant was read at 260 nm (in the case of polyA, polyG and polyU) or at 280 nm (in the case of polyC) [16].

Effect of pH, Temperature and tRNA Concentration on Ribonuclease Activity of *P. ostreatus* RNase

The effect of pH was tested using the following buffers with pH values at 37 °C,: sodium acetate, pH 3.5, 4.5 and 5.5; MES, pH 6.0, 6.5 and 7.0; HEPES, pH 7.0, 8.0 and 9.0.

A solution of *P. ostreatus* RNase (4.85 nM) in Tris-HCl buffer (pH 7.0) was incubated at 37 °C for 15 min with tRNA concentrations ranging from 0.17 to 1260 mg/ml followed by determination of the production of acid-soluble substances.

Inhibitory Activity in Cell-free Translation using a Rabbit Reticulocyte Lysate System

An assay based on the rabbit reticulocyte lysate translation system was used. Ten microlitres of the RNase was added to 10 μl of radioactive mixture (500 mM KCl, 5 mM $MgCl_2$ 130 mM phosphocreatine and 1μCi L-[4,5-^{3}H] leucine) and 30 μl working rabbit reticulocyte lysate containing 0.1 μM hemin and 5 μl creatine kinase. The reaction mixture was incubated at 37 °C for 30 min, followed by addition of NaOH and H_2O_2. After further incubation for 10 min to allow decolorization and tRNA digestion, protein with radioactive leucine incorporated was precipitated with 40% trichloroacetic acid. The precipitate was collected, washed and dried. The filter was suspended in scintillant and counted [16].

RESULTS

Purification and *N*-terminal Sequence

Ion exchange chromatography of a crude extract of *P. ostreatus* fruiting bodies on CM-Sepharose yielded a large and sharp unadsorbed peak and three adsorbed peaks labelled CM1, CM2 and CM3 (Figure 1). The first and also the smallest adsorbed peak, CM1, which possessed ribonuclease activity, was subjected to ion exchange chromatography on Mono S by FPLC. It resulted in an unadsorbed peak which was large and sharp with a tailing region, and three smaller adsorbed peaks. RNase activity was concentrated in the last peak (Figure 2) which was then fractionated by FPLC-gel filtration on Superdex

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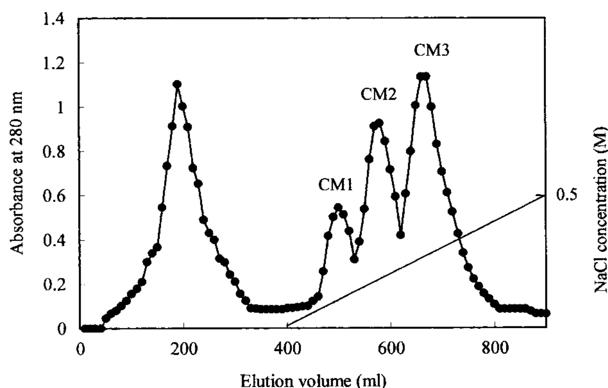

Figure 1 Cation exchange chromatography of a crude extract of fruiting bodies of *Pleurotus ostreatus* on a CM-Sepharose column (2.5 × 18 cm). The starting buffer was 20 mM NH_4OAc buffer (pH 4.6). The slanting line indicates application of a linear NaCl gradient (0–0.5 M) in the starting buffer. Ribonuclease activity was concentrated in fraction CM1.

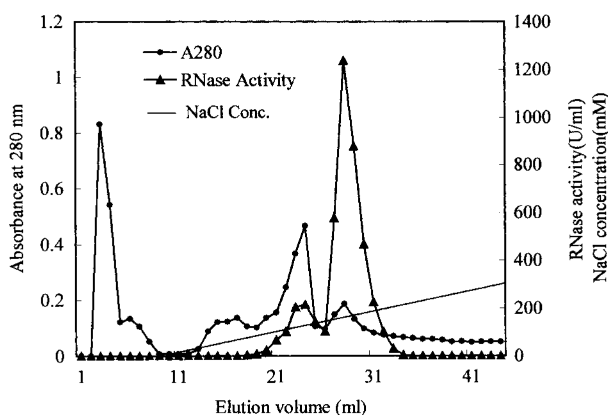

Figure 2 Ion exchange chromatography of fraction CM1 (from CM-Sepharose column) on a Mono S column by FPLC. The starting buffer was 20 mM NH_4OAc buffer (pH 4.6). The slanting line indicates application of a linear NaCl gradient (0–0.3 M) in the starting buffer. RNase activity was eluted with an elution volume of 26–32 ml.

75 into a small peak, followed by a big sharp peak and three small peaks (Figure 3). The third peak contained RNase activity. This peak demonstrated a single band with a molecular mass of 12 kDa in SDS-polyacrylamide gel electrophoresis (Figure 4). The *N*-terminal sequence of the purified RNase was APPDERTPL. It bore no resemblance to those of other mushroom RNases (Table 1).

RNase Activity of Isolated Protein

The yields and RNase activities of the various chromatographic fractions are shown in Table 2.

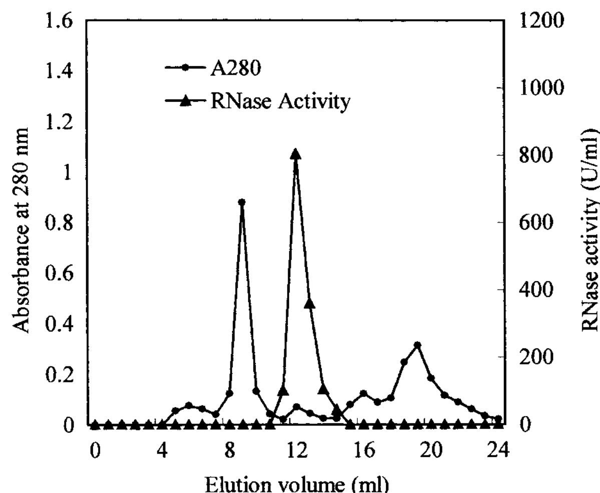

Figure 3 Gel filtration of fraction (pooled from fractions with an elution volume of 26–32 ml from the Mono S column) on a Superdex 75 column by FPLC. The buffer was 0.2 M NH_4HCO_3 buffer (pH 8.6). Fraction size: 0.8 ml. Flow rate: 0.4 ml/min. Molecular mass of the *Ostreatus Pleurotus* RNase peak observed: 12 kDa. The molecular mass standards bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen (25 kDa), cytochrome c (12.4 kDa), aprotinin (6.5 kDa), and cytidine (0.246 kDa) were used to calibrate the column.

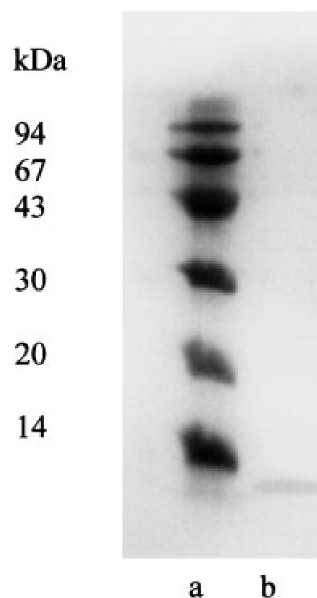

Figure 4 SDS-polyacrylamide gel electrophoresis. Left lane (lane a): Pharmacia molecular mass standards (from top downward, phosphorylase b, 94 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; soybean trypsin inhibitor, 20 kDa and lactalbumin, 14.4 kDa); Right lane (lane b): *Pleurotus ostreatus* RNase.

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Table 1 *N*-terminal Sequence of *Pleurotus ostreatus* Ribonuclease (A) in comparison with RNases from *Pleurotus ostreatus* (B), (Nomura *et al.*'s preparation) *Pleurotus ostreatus* (C), (Ye's preparation) *Pleurotus tuber-regium* (D), *Volvariella volvacea* (E), *Irpex lacteus* (F) and *Lentinus edodes* (G)

A	APPDFRTPL
B	ETGVRSCND
C	GPCYLVAFY
D	ALTAQDNRV
E	APYVQLFRP
F	VNSGCGTSG
G	ISSGCGTTG

Table 2 Ribonuclease Activity of *Pleurotus ostreatis* Chromatographic Fractions (from 225 g Fruiting Bodies) toward Yeast tRNA

Chromatographic fraction	RNase activity (U/mg)	Yield (mg)
Crude extract	49	3096
CM	238	20
Monos peak 4	3607	1.6
Superdex 75 peak 3	11 490	0.06

Ve, elution volume. The Superdex 75 chromatographic fraction with Ve = 13–15 ml represents purified *Ostreatis pulmonarius* RNase.

The optimal pH for the RNase was 7.0 and the optimal temperature was 40 °C. The RNase activity of the enzyme increased as the concentration of yeast tRNA increased to 2.5 mg/ml beyond which the curve started to level off. The RNase exhibited the highest activity toward poly U, followed by poly A and poly C. There was virtually no activity toward poly G (Table 3). The RNase elicited a dose-dependent inhibition of cell-free translation in the rabbit reticulocyte lysate system with an IC_{50} of 240 nM (Table 4).

Table 3 Activity (U/mg) of *Pleurotus ostreatus* RNase toward Poly A, Poly C, Poly G and Poly U

Poly A	Poly C	Poly G	Poly U
104	98	Undetectable	198

Table 4 Inhibition of Cell-free Translation by *Pleurotus ostreatus* RNase

Concentration (nM)	% Inhibition (mean ± SD, $n = 3$)
1.5	6.4 ± 1.4
60	372 ± 1.9
240	50.8 ± 2.4
960	89.4 ± 0.8

IC_{50} = 240 nM.

DISCUSSION

Although the *Pleurotus ostreatus* RNase purified in the present study is similar in molecular mass to the RNase prepared from the same species by Nomura *et al.* [11] they are distinct entities because the preparation of Nomura *et al.* [11] is a G-specific RNase whereas the RNase procured in the present investigation is devoid of activity toward poly G. The *N*-terminal amino acid sequences of the two RNase preparations do not show any common features either. The sequence APPDFRTPL of the present preparation contrasts with the sequence of the preparation of Nomura *et al.* [11]. The former sequence is not located in any part of the sequence of the latter preparation [11] or previously published mushroom RNases [9,10,12,13].

The present RNase preparation was adsorbed on CM-Sepharose and Mono S. The 9 kDa peptide with RNase activity isolated from the same mushroom by us [14] was also adsorbed on these two types of ion exchangers. However, the RNase isolated in the present study had a much higher RNase activity (11 490 vs 651 U/mg) and a lower translation-inhibiting activity (IC_{50} = 240 nM vs 5 nM) than the RNase reported earlier [14].

The present RNase preparation exhibits a pH optimum of 7.0 and temperature optimum of 40 °C. This compares with a pH of 6.5 and 6.5–7.5 required for optimal enzyme activity of RNase from *Pleurotus tuber-regium* [12] and *Volvariella volvacea* [13] respectively. The present RNase preparation displays a specific activity approximately one-third

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of that of *P. tuber-regium* RNase (39 000 U/mg) and two-and-a-half fold of that of *Volvariella volvacea* RNase (470 U/mg).

The present RNase preparation is endowed with an ability to inhibit translation in a cell-free reticulocyte lysate system. The activity is less potent than those exhibited by *P. tuber-regium* and *Volvariella volvacea* RNases [12,13], and lower than those of ribosome inactivating proteins that are well known with regard to their translation-inhibiting activity [17]. The cell-free translation-inhibiting activity of RNases may be ascribed, at least in part, to their ability to break down RNA which is essential for protein synthesis.

A protein with RNase and translation-inhibitory activities and an *N*-terminal sequence manifesting striking similarity to ubiquitin has been isolated from *P. ostreatus* [18]. When taken together, the present and previous [11,19] findings indicate multiplicity of proteins with RNase activity in *P. ostreatus* fruiting bodies.

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REFERENCES

1. Sasso MP, Carsana A, Confalone E, Cosi C, Somentino S, Viola M, Palmieri M, Russo E, Furia A. Molecular cloning of the gene encoding the bovine brain ribonuclease and its expression in different regions of the brain. *Nucleic Acids Res.* 1991; **19**: 6469–6474.
2. Nogues MV, Vilanova M, Cuchillo CM. Bovine pancreatic ribonuclease A as a model of an enzyme with multiple substrate binding sites. *Biochim. Biophys. Acta* 1995; **1253**: 16–14.
3. Hofsteenge J, Matthies R, Stone SR. Primary structure of a ribonuclease from porcine liver, a new member of the ribonuclease superfamily. *Biochemistry* 1989; **28**: 9806–9813.
4. Irie M, Nitta R, Ohgi K, Niwata Y, Watanabe H, Iwama M, Beintema JJ, Sanda A, Takizawa Y. Primary structure of a nonsecretory ribonuclease from bovine kidney. *J. Biochem. (Tokyo)* 1988; **104**: 289–296.
5. Liao YD. A pyrimidine-guanine sequence-specific ribonuclease from *Rana catesbiana* (bullfrog) oocytes. *Nucleic Acids Res.* 1992; **20**: 1371–1377.
6. Shapiro R, Vallee BL. Human placental ribonuclease inhibitor abolishes both angiogenic and ribonucleolytic activities of angiogenin. *Proc. Natl Acad. Sci. USA* 1987; **84**: 2238–2241.
7. Adinolfi BS, Cafaro V, D'Alessio G, Di Donato A. Full antitumor action of recombinant seminal ribonuclease depends on the removal of its *N*-terminal methionine. *Biochem. Biophys. Res. Commun.* 1995; **213**: 525–532.
8. Green PJ. The ribonucleases of higher plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 1994; **45**: 421–445.
9. Watanabe H, Hamid F, Iwami M, Onda T, Ohgi K, Irie M. Primary structure of RNase from *Irpex lacteus*. *Biosci. Biotechnol. Biochem.* 1995; **59**: 2092–2103.
10. Kobayashi H, Inokuchi N, Koyama T, Watanabe H, Iwami M, Ohgi K, Irie M. Primary structure of the nonspecific and adenylic acid preferential ribonuclease from the fruit bodies of *Lentinus edodes*. *Biosci. Biotechnol. Biochem.* 1992; **55**: 2003–2010.
11. Nomura N, Inokuchi N, Kobayashi H, Koyama T, Iwama M, Ohgi K, Irie M. Purification and primary structure of a new guanylic acid — specific ribonuclease from *Pleurotus ostreatus*. *J. Biochem. (Tokyo)* 1994; **116**: 26–33.
12. Wang HX, Ng TB. Purification and characterization of a potent homodimeric guanine-specific ribonuclease from fresh mushroom (*Pleurotus tuber-regium*) scleotia. *Int. J. Biochem. Cell Biol.* 2001; **33**: 483–490.
13. Wang HX, Ng TB. Isolation of a new ribonuclease from fresh fruiting bodies of the straw mushroom. *Biochem. Biophys. Res. Commun.* 1999; **264**: 714–718.
14. Ye XY, Ng TB. A novel peptide with ribonuclease and translation-inhibitory activity from fruiting bodies of the oyster mushroom *Pleurotus ostreatus*. *J. Peptide Sci.* 2002; **8**: 235–240.
15. Laemmli UK, Favre M. Maturation of the head of bacteriophage T4. I. DNA packaging events. *J. Mol. Biol.* 1970; **80**: 575–599.
16. Lam SSL, Wang HX, Ng TB. Purification and characterization of novel ribosome inactivating proteins, alpha- and beta-pisavins, from seeds of the garden pea *Pisum sativum*. *Biochem. Biophys. Res. Commun.* 1998; **253**: 135–142.
17. Mock JWY, Ng TB, Wong RNS, Yao QZ, Yeung HW, Fong WP. Demonstration of ribonuclease activity in the plant ribosome-inactivating proteins alpha- and beta-momorcharins. *Life Sci.* 1996; **59**: 1853–1859.
18. Barbieri L, Battelli MG, Stirpe F. Ribosome-inactivating proteins from plants. *Biochim. Biophys. Acta* 1993; **1154**: 237–282.
19. Wang HX, Ng TB. 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.* 2000; **276**: 587–593.

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