Purification and Characterization of a Nuclease from *Lentinus edodes*

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An endonuclease with 3'-nucleotidase activity (nuclease Le1) was purified from fruit bodies of *Lentinus edodes* in a single band on sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The apparent molecular weight of nuclease Le1 was about 27000. The nuclease was inactivated in the presence of ethylenediaminetetraacetic acid (EDTA) and reactivated by the addition of Zn^{2+} . Hydrolysis of poly U by the nuclease showed many intermediate size oligomers prior to the formation of 5'-uridine monophosphate (UMP). Therefore, it was concluded that nuclease Le1 was a Zn^{2+} -endonuclease similar to P1-nuclease from *Penicillium citrinum*. The nuclease was very sensitive to ionic strength, but pH-profiles of the hydrolysis of four 3'-nucleotides were very similar to those of P1 nuclease from *P. citrinum*.

Keywords nuclease; 3'-nucleotidase; *Lentinus edodes*; purification; amino acid composition; zinc-reactivation; molecular weight

5'-Guanylic acid is known as the major component of the tasty substance in *Lentinus edodes*. It is evident that the production of this compound is correlated to the nucleic acid metabolism in *L. edodes*, in which several ribonucleic acid (RNA) degrading enzymes are reportedly present.^{1–4} In a previous paper, we isolated one of the enzymes, ribonuclease (RNase) Le2, from the fruit bodies of *L. edodes* and characterized it as a base non-specific and adenylic acid preferential RNase.⁵ In this paper, we report the purification of another RNA degrading enzyme from *L. edodes* and characterize it as a Zn^{2+} -endonuclease with 3'-nucleotidase activity.

Materials and Methods

Substrate Yeast RNA is a product of Kojin Co. (Tokyo). 3'-Nucleotides, 5'-nucleotides and salmon sperm deoxyribonucleic acid (DNA) were the products of Wako Pure Chemicals (Osaka). Homopoly-nucleotides were the products of Yamasa (Chiba).

Reagents Molecular weight marker proteins were obtained from Oriental Yeast Co. (Tokyo). The reagents for polyacrylamide gel electrophoresis (PAGE) were purchased from Wako Pure Chemicals. Phospho-cellulose and diethylaminoethyl (DEAE)-cellulose were obtained from Serva (West Germany). Sephadex G-50, and SP-Sephadex C-50 were obtained from Pharmacia (Uppsala). DEAE-toyopearl 650M, Butyl-toyopearl, QAE-toyopearl 550C and TSK-gel G2000SW for high performance liquid chromatography (HPLC) were the products of Toso (Tokyo).

Enzyme Source Fresh fruit bodies of *L. edodes* were supplied by Mori Mushroom Research Institute (Kiryu). Alkaline phosphatase from *Escherichia coli* was purchased from Wako Pure Chemicals.

Enzyme Assay (a) RNase assay was carried out as described in the previous paper⁵ with RNA as substrate at pH 4.75 and 37°C. The enzyme unit was defined as the absorbancy increase per 5 min under the experimental conditions described above.

(b) Hydrolyses of DNA, homopolynucleotides, and 3'- or 5'-nucleotides were followed by the method of Fujimoto *et al.*⁶ Twenty mM of buffer (0.4 ml) containing 1 mg nucleic acid or nucleotide was treated with 10 μ l of enzyme solution at 37°C for 10 min. The reaction was terminated by the addition of an equal volume of 0.2M Tris-HCl buffer (pH 9.1). To the reaction mixture was added 0.25 unit of alkaline phosphatase and the release of Pi was measured as described by Chen *et al.*⁷

Protein Concentration Protein concentration was measured colorimetrically according to Smith *et al.*⁸ using bovine serum albumin as standard.

Sodium dodecylsulfate (SDS)-PAGE SDS-PAGE was conducted in 12.5% polyacrylamide gel according to the method of Laemmli.⁹ The gel was stained with a silver staining kit (Daiichi Kagaku, Tokyo). Molecular weight of nuclease was determined by SDS-PAGE using molecular weight marker proteins.

Isoelectric Focusing Electrofocusing on 5% polyacrylamide gel was performed according to the procedure of Dale and Latner¹⁰ using ampholine covering pH 3.5–10.0 after overnight electrophoresis at 200 V; voltage was then raised to 400 V for 2 h. The pI value of nuclease was estimated by comparing the mobility of nuclease with that of pI markers (pI 4.1–10.6).

Amino Acid Analysis Amino acid analysis was done by the method of Bidlingmeyer *et al.*¹¹ with a Pico TagTM amino acid system (Waters). Phenylthiocarbamoyl amino acids were separated by reversed phase HPLC with a Pico-TagTM column. The separation conditions were previously described.⁵ Tryptophan content was determined by the method of Pajot.¹²

Edman Degradation Nuclease Le1 was subjected to automated Edman degradation using an Applied Biosystem 477A protein sequencer with a 120A PTH-analyzer according to Hewick *et al.*¹³

Hydrolysis of RNA with a Purified Nuclease RNA (0.5 mg) was dissolved in 50 mM acetate buffer (pH 4.75) and incubated with 5 μ g of enzyme at 37°C. Aliquots were withdrawn at appropriate intervals and chromatographed as described.⁵

Time Course of Hydrolysis of Poly U Checked by Gel-Filtration To 0.2 ml Poly U solution containing 0.5 mg poly U, 5 μ l of enzyme (*ca.* 0.5 u) was added. The reaction mixture was incubated at 37°C. Fifty μ l aliquots were withdrawn at an appropriate time and injected to the column of TSK-gel G2000SW equilibrated with 50 mM ammonium acetate buffer (pH 6.0). The column was eluted with the same buffer at the flow rate of 0.5 ml/min. The eluate was monitored by the absorbancy at 260 nm.

Zn^{2+} Content Zn^{2+} content in nuclease Le1 was determined with a Shimadzu atomic absorption spectrometer A 660 at 213.8 nm using 3 nmol of nuclease.

Results

Purification of RNA Hydrolyzing Enzyme We previously found two RNA hydrolyzing enzymes in the fresh fruit bodies of *L. edodes*.⁵ One of them was identified as the base non-specific and adenylic acid preferential RNase.⁵ In this paper, we purified another enzyme. During the course of purification described below, 0.1 mM $ZnCl_2$ was added to the buffers used.

Step 1: The crude extract of fresh fruit bodies of *L. edodes* (1.5 kg) was extracted with 3.75 l of 10 mM sodium acetate buffer (pH 6.0) as described.⁵

Step 2: To 6 l of crude extract, 600 g of DEAE-cellulose (wet weight) pre-equilibrated with 10 mM sodium acetate buffer (pH 6.0) was added and the suspension was stirred gently at 0°C for 30 min. The DEAE-cellulose-adsorbed protein was collected by suction and washed with 3 l of the buffer, then suspended in the same buffer. The column packed with the DEAE-cellulose was eluted with a linear

Fig. 1. DEAE-Cellulose Column Chromatography of Crude Extract

The crude extract of step 1 was adsorbed on a column (6 × 40 cm) of DEAE-cellulose equilibrated with 10 mM sodium acetate buffer (pH 6.0). After washing with 3 l of the same buffer, the column was eluted with a linear gradient of 0–1 M NaCl in 4 l of the same buffer. Fifteen ml fractions were collected, and those indicated by a heavy bar were pooled. —, A_{280} ; ○, RNase activity; — — —, NaCl concentration.

gradient of 0–1 M NaCl in 4 l of the same buffer. Fifteen ml fractions were collected (Fig. 1). The enzyme activity was separated into two fractions, the first (tube Nos. 70–99, RNase Le1 fraction) and the second (tube Nos. 100–180, RNase Le2 fraction).

Step 3: The RNase Le1 fraction was concentrated *in vacuo*, then dialyzed against deionized water. The pH of the dialyzate was adjusted to 6.0, and the precipitate formed was eliminated by centrifugation at 10000 rpm for 15 min. The supernatant was fractionated by gel-filtration on Sephadex G-50 (4 × 210 cm, Pharmacia) equilibrated with 10 mM sodium acetate buffer (pH 6.0). Ten ml fractions were collected. The active RNase fractions (tube Nos. 55–80) were pooled.

Step 4: SP-Sephadex C-50 Column Chromatography: The active RNase fraction of step 3 was dialyzed against deionized water. The pH of the dialyzate was adjusted to 5.0 and put on a column of SP Sephadex C-50 (3 × 30 cm, Pharmacia) equilibrated with 10 mM sodium acetate buffer (pH 5.0). Ten ml fractions were collected. The enzymatic activity was found in the unadsorbed fraction (tube Nos. 22–33).

Step 5: DEAE-Toyopearl 650M Column Chromatography: The active RNase fraction of step 4 was dialyzed against deionized water. The pH of dialyzate was adjusted to 6.0 and put on a column of DEAE-toyopearl 650M (1 × 25 cm, Tosoh) equilibrated with 10 mM sodium acetate buffer (pH 6.0). The column was eluted with a linear gradient of 200 ml of 10 mM sodium acetate buffer (pH 6.0) and 200 ml of sodium acetate buffer (pH 6.0) containing 0.3 M NaCl. Three ml fractions were collected. Active RNase fractions were pooled (tube Nos. 54–60).

Step 6: Butyl-Toyopearl Column Chromatography: The active RNase fraction of step 5 was dialyzed against 0.1 M phosphate buffer (pH 6.5) and solid ammonium sulfate was added to the dialyzate to make 1.2 M. The enzyme solution was applied on a column of Butyl-toyopearl (1 × 20 cm) equilibrated with the same buffer described above but containing 1.2 M ammonium sulfate (200 ml). The column was eluted with a linear gradient of 200 ml of the equilibrating buffer and the same buffer without ammonium sulfate. Three ml fractions were collected. The active

TABLE I. Purification of Nuclease Le1 from the Caps of Fruit Bodies of *L. Edodes* (1.5 kg)

Step	Total activity (unit)	Total protein (mg)	Specific activity (unit/mg)	Yield (%)
1. Crude extract	5940	34900	0.17	100
2. DEAE-cellulose	1420	2700	0.52	23.9
3. Sephadex G-50	1250	781	1.60	21.0
4. SP-Sephadex	760	281	2.70	12.8
5. DEAE-toyopearl	428	6.40	66.8	7.2
6. Butyl-toyopearl	214	2.69	79.6	3.6
7. QAE-toyopearl	187	2.18	85.7	3.1
8. TSK-gel G2000SW	113	0.88	128	1.9

Fig. 2. SDS-PAGE of Nuclease Le1

SDS-PAGE of the purified RNase fraction Le1 (nuclease Le1) stained with silver staining kit. Lane 1, standard proteins of molecular weight 74000, 49600, 37200, 24800, 12400; lane 2, RNase fraction Le1.

TABLE II. Effect of Divalent Cations and EDTA on the Enzymatic Activity of Nuclease Le1

Addition	Enzymatic activity (%)
None	100 (27)
Zn ²⁺	100 (89)
Mg ²⁺	100 (31)
Mn ²⁺	100 (28)
Ca ²⁺	100 (27)
Fe ²⁺	98 (27)
Sr ²⁺	98 (25)
Ba ²⁺	98 (24)
Cd ²⁺	85 (18)
Ni ²⁺	75 (17)
Hg ²⁺	37 (12)
Cu ²⁺	39 (8)
EDTA	27 (27)

The figures in parentheses indicate the effect of divalent cation on the enzyme pre-treated with EDTA (27% activity).

RNase fraction (tube Nos. 89–100) was collected.

Step 7: QAE-Toyopearl 550C Column Chromatography: The active RNase fraction of step 6 was dialyzed against deionized water, and the pH of the solution was adjusted to 6.0. The enzyme solution was applied on a column of QAE-toyopearl (1 × 20 cm). The column was eluted with the linear gradient of between 200 ml of the same buffer and the same buffer containing 0.25 M NaCl. Three ml fractions were collected. The active RNase fractions (tube Nos. 121–126) were pooled.

Step 8: HPLC on TSK-Gel G2000SW: The active RNase

Fig. 3. Effect of pH on the Rate of Hydrolysis of RNA, DNA, Homopolynucleotides and Four 3'-Nucleotides by Nuclease Le1

Substrate concentration: 2.5 mg/ml. Enzyme concentration: 0.002–0.13 u. Buffers used were: 0.1 M citrate buffer for pH 3–6.5, 0.1 M Mes buffer for pH 6–7, 0.1 M Tris-HCl buffer for pH 7–9, 0.1 M glycine-NaOH buffer for pH 9–11.5 and KCl-NaOH for pH 12–13. (a) ●, RNA; ○, heat denatured DNA; ×, double stranded DNA. (b) ●, poly A; ○, poly U; ▲, poly G; △, poly C. (c) ●, 3'-AMP; ○, 3'-UMP; ▲, 3'-GMP; △, 3'-CMP.

Fig. 4. Time Course of Degradation of Poly U by Nuclease Le1

The digestion products of poly U by nuclease Le1 were separated by gel filtration on TSK-gel G2000SW. —, 0 time; ---, 1 min; - - -, 2 min; — · —, 5 min; — · · —, 30 min. Arrow at retention time 21.6 min indicates the elution position of 5'-UMP. Other experimental conditions were as described in Materials and Methods.

Fig. 5. Hydrolysis Products of RNA by Nuclease Le1

The hydrolysis products of 24 h were applied to two-dimensional paper chromatography. Solvents used for the first and second dimensions were isobutyric acid; 0.5 N NH_4OH (10:6, v/v) and 2-propanol: water: saturated ammonium sulfate in water: 0.1 M sodium citrate-phosphate buffer (pH 6.0) (2:80:20, v/v), respectively. Dark spot, GMP; heavy dotted spot, AMP; hatched spot, UMP and light dotted spot, CMP.

fraction of step 7 was concentrated to 0.2 ml and gel-filtrated with TSK-gel G2000SW HPLC (0.78 × 30 cm) equilibrated with 50 mM ammonium acetate buffer (pH 6.0), and eluted with the same buffer. Five-tenth ml fractions were collected. The active RNase fractions (tube Nos. 17–20) were pooled.

The RNase fraction (RNase Le1) was purified 753 fold, at a yield of ca. 2%. Purification procedures are summarized in Table I. The RNase Le1 thus purified gave a single band on SDS-PAGE (Fig. 2).

Effect of Divalent Cations and EDTA At 1 mM concentration, among the various divalent cations and EDTA tested with RNA as substrate, Cu^{2+} , Hg^{2+} inhibited markedly the RNase activity and inhibited moderately the Ni^{2+} and Cd^{2+} . However, the RNase activity was inhibited by EDTA. The EDTA inactivated enzyme was markedly reactivated with Zn^{2+} (Table II). This evidence seems to suggest that the RNase Le1 fraction is not a simple RNase, but a Zn^{2+} -enzyme like P1 nuclease from *Penicillium citrinum*,¹⁴⁾ and S1 nuclease from *Saccharomyces cer-*

visiae.¹⁴⁾

Substrate Specificity In order to characterize the RNase Le1, the enzymatic activities towards DNA, homopolynucleotides, 3'-nucleotides and 5'-nucleotides were measured at various pHs. RNase Le1 fraction hydrolyzed heat-denatured DNA as well as RNA, but not double stranded DNA, though the optimum pH for DNA cleavage was somewhat different from that of RNA (Fig. 3). This evidence revealed Le1 to be a nuclease specific for single stranded RNA and DNA. The enzyme was active towards 3'-nucleotides but not 5'-nucleotides (data not shown). The rate of hydrolysis of RNA is about 1.45 fold that of single stranded DNA. Since this enzyme works both for DNA and RNA, we renamed it nuclease Le1.

As shown in Fig. 4, when poly U was hydrolyzed by nuclease Le1, various sizes of polynucleotides were observed

before the formation of 5'-uridine monophosphate (UMP). It was therefore, evident that the nuclease was an endonuclease with 3'-nucleotidase activity.

Nuclease Le1 hydrolyzed RNA and accumulated four 5'-nucleotides (Fig. 5). Thus it was a 5'-former nuclease.

Other Properties of Nuclease Le1 Molecular weight of nuclease Le1 estimated from SDS-PAGE was about 27000 (Fig. 2). The pI of nuclease Le1 obtained from disc gel electrophoresis¹⁰⁾ was 4.6 (data not shown). As shown in Fig. 6, the nuclease Le1 activity was markedly inhibited by the increase of ionic strength as compared to that of RNase Le2 coexisting in *L. edodes*.⁵⁾ When we incubated the nuclease at 37°C for 1 h, nuclease Le1 was stable between pH 5–6, and became unstable below pH 4 and above 7.0 (Fig. 7). Zn^{2+} Content of nuclease Le1 contains 1.5–2.0 atoms of Zn^{2+} per mol enzyme. From this evidence and the results described above, it was evident that nuclease

Fig. 6. Effect of Salt Concentration on Nuclease Le1 Activity

Details of the experimental conditions are the same as the assay method (a) with RNA as substrate. ●, nuclease Le1; ○, RNase Le2.

Fig. 7. pH Stability of Nuclease Le1

Nuclease Le1 (0.6 u) was incubated at various pH and 37°C for 1 h, then 1/10 volume of 2 M acetate buffer (pH 4.75) was added to the reaction mixtures. The enzymatic activities of each incubation mixture were measured with the assay method (a) at pH 4.75.

Fig. 8. N-Terminal Amino Acid Sequence of Nuclease Le1

The sequence of nuclease from *P. citrinum* is also included. Common sequences between these two enzymes are blocked.

Le1 was a Zn^{2+} endonuclease.

Amino Acid Composition of Nuclease Le1 The amino acid composition of nuclease Le1 is shown in Table III and that of a typical nuclease, P1 nuclease is shown for comparison. Half cystine content was the same as that of P1 nuclease. The results also show the similarity between these two enzymes.

Amino-Terminal Sequence of Nuclease Le1 The amino terminal amino acid sequence of nuclease Le1 was determined by Edman degradation (Fig. 8). The amino terminal sequence is very similar to that of *Penicillium* enzyme⁵⁾ indicating the homogeneity of nuclease. The sequence homology between two enzymes of the first 19 residues so far determined is 63%.

Discussion

Accumulation of 5'-nucleotides during the processing of edible mushrooms was observed by Mouri *et al.*,¹⁾ and they suggested the participation of some RNA cleaving enzymes in the production of these nucleotides. Kadowaki *et al.*²⁾ also described the presence of nuclease which forms 5'-nucleotides in dry shii-take (*L. edodes*) fruit body. Endo *et al.*³⁾ partially purified three 3'-nucleotidase fractions from shii-take, and reported their properties. In this paper, we purified only one nuclease with 3'-nucleotidase activity in the homogeneous state as judged by SDS-PAGE. The enzymatic properties of this purified nuclease, such as pH optimum and pH-activity profile of 3'-nucleotidase towards

TABLE III. Amino Acid Composition of Nuclease Le1

Amino acid	Experimental value ^{a)}	Nuclease from <i>Penicillium</i> sp. ^{b)}
Trp	3.8 (4)	9
Lys	5.9 (6)	7
His	5.7 (6)	11
Arg	9.0 (9)	6
Asp	21.0 (21)	32
Thr	15.5 (16)	17
Ser	22.5 (23)	29
Glu	24.6 (25)	22
Pro	18.3 (18)	8
Gly	18.3 (18)	21
Ala	30.5 (31)	34
1/2Cys	4.0 (4)	4
Val	19.4 (19)	4
Met	4.2 (4)	3
Ile	15.9 (16)	18
Leu	20.4 (20)	18
Tyr	8.6 (9)	14
Phe	12.9 (13)	4
Total	263	270

a) Calculated assuming Arg residue as 9.0. The amino acid contents of Ser, Thr, and Met are the values extrapolated to zero time from the data of 24 and 48 h hydrolysis. Parenthesis indicates the nearest integer. b) Amino acid composition of the nuclease from *Penicillium* sp. was obtained from the literature.¹⁸⁾

four 3'-nucleotides, were essentially the same as those of the three 3'-nucleotidase fractions reported by Endo *et al.*³⁾ The production of three species of nuclease in dry shii-take is probably due to some processing of 3'-nucleotidase during the drying sequence. As shown above, nuclease Le1 is a Zn^{2+} enzyme, so we added 0.1 mM ZnCl_2 to the buffers used for purification to prevent the loss of the enzyme.

As described above, we showed that nuclease Le1 contains about 2 atoms of Zn^{2+} . Therefore, it belonged to the same category as P1 nuclease and S1 nuclease found in fungi.^{14,15)} As shown in Fig. 3, nuclease Le1 liberated inorganic phosphate from 3'-nucleotides, but not from 5'-nucleotides. The optima for three 3'-nucleotides, 3'-UMP, 3'-cytidine monophosphate (CMP) and 3'-adenosine monophosphate (AMP) was 7.0–7.5 and that of 3'-guanosine monophosphate (GMP) was about 10.0. The results shown in this figure are very similar to those observed for P1 nuclease.¹⁶⁾ Thus, nuclease Le1 seems to be a very similar enzyme as P1 nuclease.

The primary structures of this kind of nuclease were determined by Iwamatsu *et al.*¹⁷⁾ only for *Saccharomyces cerevisiae* nuclease (S1 nuclease) and by Tabata *et al.*¹⁸⁾ and Maekawa *et al.*¹⁹⁾ for *P. citrinum* and related species. The N-terminal sequence of nuclease Le1 is very similar to that of the *Penicillium* enzyme, and the sequence homology is 63%. But even in unidentical amino acid residues, we observed several substitution in these two nucleases between amino acid of similar nature, such as Leu to Val, Phe to Tyr and Ile to Leu. Since other fungi such as *Flammulina velutipes* contain similar nucleases,²⁰⁾ elucidation of the structure of nuclease Le1 might provide useful information on their mechanism of action and also on the chemotaxonomy of fungi.

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