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A Novel Ribonuclease with Antiproliferative Activity from Fresh Fruiting Bodies of the Edible Mushroom *Lyophyllum shimeiji*

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Abstract A 14.5-kDa ribonuclease, with an optimum pH of 6 and a temperature optimum at 70°C, was isolated from fresh fruiting bodies of the edible mushroom *Lyophyllum shimeiji*. It was purified by ion exchange chromatography on DEAE cellulose, Q Sepharose, and SP Sepharose, followed by FPLC gel filtration on Superdex 75, and was adsorbed on all three ion exchangers. It showed the highest ribonucleolytic potency toward poly (U), 25% as much activity toward poly (C), and undetectable activity toward poly (A) and poly (G). Its ribonucleolytic activity at 100°C was similar to that at 20°C. It suppressed proliferation of hepatoma HepG2 cells and breast cancer MCF7 cells with an IC_{50} of 10 and 6.2 μM, respectively. It inhibited the activity of HIV-1 reverse transcriptase with an IC_{50} of 7.2 μM.

Keywords Antiproliferative · *Lyophyllum shimeiji* · Mushroom · Ribonuclease

Introduction

Ribonucleases (RNases) are present in a diversity of organisms comprising bacteria (Hartley 1988; Zilhao et al. 1993), mushrooms (Kobayashi et al. 1992; Ng 2004; Ng and Wang 2004; Ngai and Ng 2004; Nomura et al. 1994; Wang and Ng 1999, 2001, 2003a, b, c, 2004a, b; Ye and Ng 2002a, b), flowering plants (Green 1994; Lam and

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Ng 2001b; Ng and Wang 2001; Wang and Ng 2000), and vertebrate animals (Adinolfi et al. 1995; Hofsteenge et al. 1989; Irie et al. 1988; Liao 1992; Sasso et al. 1991). In vertebrate animals, RNases have been reported from the brain (Sasso et al. 1991), kidney (Irie et al. 1988), liver (Hofsteenge et al. 1989), semen, and milk (Adinolfi et al. 1995; Matousek et al. 1995; Shapiro and Vallee 1987). Some of the ribonucleases demonstrate immunosuppressive antiviral (Silverman 1997), antiproliferative, and antitumor (Adinolfi et al. 1995) activities.

Mushrooms are a rich source of bioactive proteins, including lectins, antifungal proteins, ribosome inactivating proteins, and RNase (Adinolfi et al. 1995; Lam and Ng 2001a; Ng and Lam 2002; Ye and Ng 2002a, b). Different mushroom species produce RNases with different N-terminal sequences, pH, temperature optima, and polyhomoribonucleotide specificities (Kobayashi et al. 1992; Ng and Wang 2004; Ngai and Ng 2004; Nomura et al. 1994; Wang and Ng 1999, 2001, 2003a, b, c, 2004a, b; Ye and Ng 2002a, b). *Pleurotes sajor-caju* ribonuclease has a variety of activities, including antimitogenic activity toward mouse splenocytes, antiproliferative activity toward tumor cells, and growth-inhibitory activity toward fungi and bacteria (Ngai and Ng 2004). Thus, it would be worthwhile to isolate RNases from mushrooms that have not been investigated to see if they have unique characteristics. From the fruiting bodies of *Lyophyllum shimeiji*, a lectin, an antifungal protein and a ribosome inactivating protein have been isolated (Lam and Ng 2001a; Ng and Lam 2002). The purpose of the present study was to isolate and characterize an RNase from *L. shimeiji*.

Materials and Methods

Isolation of RNase

All chemicals used in this study were from Sigma Chemical Company, St. Louis, MO, USA, unless otherwise stated. Fresh edible mushroom *Lyophyllum shimeiji* (1 kg) was obtained from a local supplier of Yunnan Province, China. The fruiting bodies were homogenized in distilled water (3 ml/g). Following centrifugation of the homogenate, Tris–HCl buffer (pH 7.4, 1 M) was added to the resulting supernatant until its concentration reached 10 mM. The supernatant was fractionated by ion exchange chromatography on a 5 × 20 cm column of DEAE cellulose. After elution of unadsorbed proteins (fraction D1) with 10 mM Tris–HCl buffer (pH 7.4), adsorbed proteins were desorbed sequentially with 0.2 M NaCl and 1 M NaCl in the 10 mM Tris–HCl buffer (pH 7.4) to form fractions D2 and D3, respectively. Fraction D2 with ribonuclease activity was chromatographed on a 2.5 × 20 cm column of Q Sepharose (GE Healthcare). Unadsorbed proteins were eluted as fraction Q1, and adsorbed proteins were eluted as fractions Q2 and Q3 with a linear 0–1 M NaCl concentration gradient. Fraction Q2 with ribonuclease activity was dialyzed and subsequently applied on a 2.5 × 20 cm column of SP Sepharose (GE Healthcare). Unadsorbed proteins were eluted as fraction SP1, and adsorbed proteins were eluted as fractions SP2, SP3, and SP4 with a linear 0–1 M NaCl concentration gradient. Fraction SP3 with ribonuclease activity was dialyzed and

subsequently further purified on a Superdex 75 h 10/30 column (GE Healthcare) in 0.2 M NH_4HCO_3 buffer (pH 8.5). The second resulting peak (SU2) represented purified RNase.

Molecular Mass Determination

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out following 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. FPLC gel filtration was conducted in a Superdex 75 h 10/30 column calibrated with molecular-mass markers (GE Healthcare) using an AKTA Purifier (GE Healthcare).

Analysis of N-Terminal Amino Acid Sequence

Amino acid sequence analysis was performed 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 was determined by measuring the formation of acid-soluble, UV-absorbing species with the method of Wang and Ng (1999). The RNase was incubated with 200 μg tRNA in 150 μg of 100 mM MES buffer (pH 6) at 37°C for 1 h. The reaction was stopped by addition of 350 μl 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 OD_{260} of the supernatant was measured after appropriate dilution. One unit of enzymatic activity is defined as the amount of enzyme that brings about an increase in OD_{260} of one per minute in the acid-soluble fraction per milliliter of reaction mixture under the specified conditions.

Activity of RNase Toward Polyhomoribonucleotides

The ribonucleolytic activity of the purified RNase toward polyhomoribonucleotides was assayed with a modification of the method of Wang and Ng (2001). Incubation of RNase with 100 μg poly (A), poly (C), poly (G), or poly (U) in 250 μl of 100 mM MES buffer (pH 6.0) was performed at 37°C for 1 h, before introduction of 250 μl ice-cold 1.2 N perchloric acid containing 20 mM lanthanum nitrate to stop the reaction. After leaving 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 Antifungal Activity

Antifungal activity toward *Mycosphaerella arachidicola* and *Physalospora piricola* was assayed on 100 mm × 15 mm petri plates containing 10 ml potato dextrose

agar. After the mycelial colony had formed, sterile blank paper disks (0.625 cm in diameter) were placed at a distance of 0.5 cm from the periphery of the mycelial colony. An aliquot (15 μl) of the mushroom RNase was added to a disk. The plates were incubated at 23°C for 72 h until mycelial growth had surrounded the disks containing the control and had formed crescents of inhibition around disks containing samples with antifungal activity (Ng and Wang 2001).

Assay of Hemagglutinating Activity

The assay was conducted using rabbit erythrocytes (Wang and Ng 2005).

Assay of Antiproliferative Activity on Tumor Cell Lines

HepG2 and MCF 7 cells were cultured in RPMI medium supplemented with 10% (v/v) fetal bovine serum (Invitrogen), 100 mg/l streptomycin, and 100 IU/ml penicillin, at 37°C in a humidified atmosphere of 5% (v/v) CO_2. Cells were subsequently seeded into 96-well plates with a concentration of 2×10^3 cells/well and incubated for 24 h. Different concentrations of *L. shimeiji* RNase in 100 μl complete RPMI medium were then added to the wells and incubated for 72 h (Ngai and Ng 2004). After that MTT quantification assays were carried out to measure the cells' viability. Briefly, 20 μl of a 5 mg/ml solution of 3-(4,5-dimethythiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) in phosphate-buffered saline was spiked into each well and the plates were incubated for 4 h. The plates were then centrifuged at 2500 rpm for 5 min. The supernatant was carefully removed, and 150-μl 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 protease as control.

Assay for HIV Reverse Transcriptase Inhibitory (HIV RT) Activity

The assay for HIV RT inhibitory activity was performed, as described by Zhao et al. (2009), 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 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 (dA)-oligo (dT) 15. In this assay, nucleotides labeled with digoxigenin and biotin in an optimized proportion were incorporated into the DNA, which was 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, which had been conjugated to peroxidase, to digoxigenin (anti-DIG-POD) was then used to bind to the digoxigenin-labeled DNA. This was followed by the addition of peroxidase substrate. The peroxidase enzyme, conjugated to the antibody, then catalyzed the cleavage of the substrate to produce a colored product. The

absorbance was measured at 405 nm with a microtiter (ELISA) reader and correlated to the level of RT activity. A fixed amount (4–6 ng) of recombinant HIV-1 reverse transcriptase was used. The activity of inhibition exhibited by the *L. shimeiji* RNase was determined as compared to a control without RNase.

Results

Chromatography of the fruiting body extract on DEAE cellulose gave rise to an unadsorbed fraction D1 and an adsorbed fraction D2 containing slightly less protein than D1. Ribonuclease activity was concentrated in fraction D2 (Table 1). Fraction D2 was resolved on Q Sepharose to produce a small unadsorbed fraction Q1, with negligible RNase activity, and two larger adjacent adsorbed fractions, Q2 and Q3, of approximately the same size. Ribonuclease activity was enriched in fraction Q2 (Table 1). Fraction Q2 was separated on SP Sepharose into an unadsorbed fraction SP1 and several adsorbed fractions, SP1-SP4 (Fig. 1). The bulk of ribonuclease activity resided in fraction SP3 (Table 1). Fraction SP3 was resolved on Superdex 75 into two fractions, SU1 and SU2, of about the same size (Fig. 2). Ribonuclease activity was confined to fraction SU2 (Table 1). Fraction SU2 displayed a single band with a molecular mass of 14.5 kDa in SDS–PAGE (Fig. 3).

The ribonucleolytic activity of the purified ribonuclease increased steadily from pH 3 until it reached its maximum at pH 6 and then decreased steadily from pH 6 until it reached its residual level at pH 9 (Fig. 4). The activity fell precipitously when the temperature was raised from 80 to 90°C. The activity observed at 100°C was similar to that at 20°C (Fig. 5). The ribonuclease exerted a specific ribonucleolytic activity of 0.1 U/mg toward poly (A) and poly (G), 3.5 U/mg toward poly (C), and 14.2 U/mg toward poly (U). The N-terminal sequence of the

Table 1 Purification of *Lyophyllum shimeiji* mushroom RNase

Fraction	Yield (mg)	Specific RNase activity (U/mg)	Purification fold
Extract	2285.7	149.5	1
D1	421.1	<10	–
D2	550.4	452.9	3.0
D3	605.6	<10	–
Q1	68.8	<10	–
Q2	145.1	1226.9	8.2
Q3	168.0	80.2	–
SP1	16.2	<10	–
SP2	24.4	145.0	–
SP3	26.1	5280.8	35.3
SP4	34.5	<10	–
SU1	4.9	167.3	–
SU2	12.0	7520.9	50.3

From 1000 g fresh fruiting body

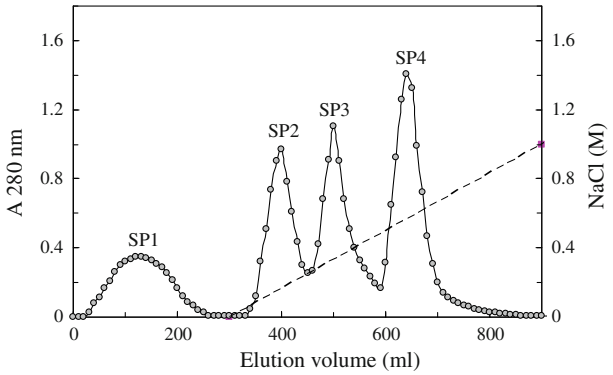

Fig. 1 Ion exchange chromatography on SP Sepharose. Sample: Fraction Q2, which was adsorbed on DEAE cellulose; column dimensions: 2.5 × 20 cm; starting buffer: 10 mM NH_4OAc (pH 5.0). *Dashed line* across the right side of the chromatogram represents linear 0–1 M NaCl gradient used to elute adsorbed proteins

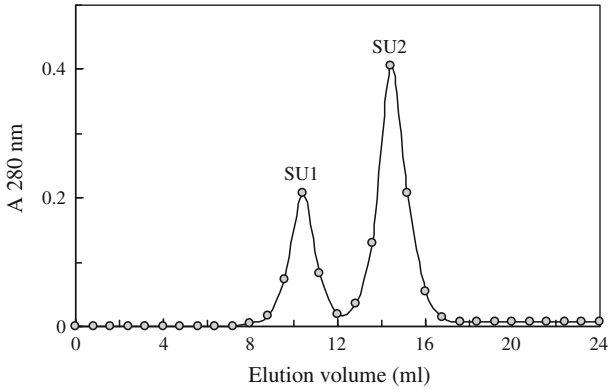

Fig. 2 FPLC gel filtration of fraction SP3 on a Superdex 75 h 10/30 column. Buffer: 0.2 M NH_4HCO_3 (pH 8.5); flow rate: 0.4 ml/min; fraction size: 0.8 ml

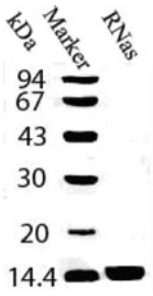

Fig. 3 SDS-PAGE of purified *Lyophyllum shimeiji* RNase; fraction SU2 (*right lane*). Marker proteins (*left lane*). Molecular mass indicated in kDa on the left

purified ribonuclease exhibited little similarity to counterparts from other mushrooms (Table 2). The protein was devoid of antifungal activity and hemagglutinating activity when tested at 300 μg.

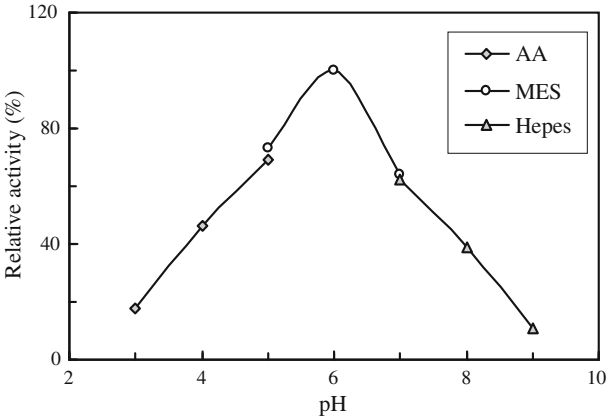

Fig. 4 *Lyophyllum shimeiji* RNase activity and pH dependence. Temperature: 37°C; duration of incubation: 15 min; buffer concentration: 0.1 M

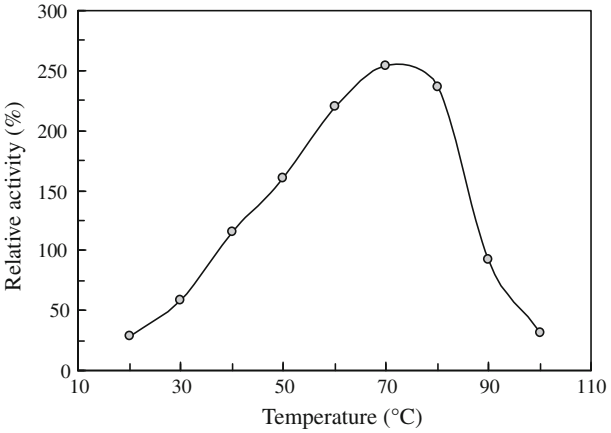

Fig. 5 *Lyophyllum shimeiji* RNase activity and temperature dependence. Buffer: pH 6.0, 0.1 M MES buffer; duration of incubation: 15 min

At the concentrations of 2.5, 5, 10, and 20 μM, the purified RNase inhibited the proliferation of HepG2 cells by 3.4, 20.1, 50.4, and 75.3% and the proliferation of MCF7 cells by 14.6, 41.7, 68.5, and 84.9%, respectively. The IC_{50} values toward HepG2 cells and MCF7 cells were 10.0 and 6.2 μM, respectively.

At the concentrations of 0.8, 4, and 20 μM, the purified RNase inhibited HIV-1 reverse transcriptase by 5.2, 37.0, and 93.4%, respectively. The IC_{50} value was 7.2 μM.

Discussion

Like most other mushroom RNases (Ye and Ng 2002a, b), *Lyophyllum shimeiji* RNase is adsorbed on a cation exchanger. It resembles RNases from *Pleurotus*

Table 2 Comparison of the N-terminal sequence of *Lyophyllum shimeiji* ribonuclease with ribonucleases from 14 other organisms

Origin	N-terminal sequence of RNase[a]	Reference
Lyophyllum shimeiji	AATCW KTSTA	This paper
Agaricus bisporus	ACAANNAARRYYASSVGNNN	Wang and Ng (2006)
Thelephora ganbajun	DADIAVWAPPVNAQN	Wang and Ng (2003a)
Clitocybe maxima	ETAHTHAGIQYSTVDVNNSIMKAVGGGAGN	Wang and Ng (2004a)
Pleurotus pulmonarius	AISANNERKGVNQQSVQNTYQENDV	Ye and Ng (2002a)
Volvariella volvacea	APYVQLFRPLIQPQVLATFAIANNMAQY	Wang and Ng (1999)
Lentinus edodes	ISSGCGTTGALSCSSNAKGTCCFEAPGGLI	Kobayashi et al. (1992)
Irpex lacteus	VNSGCGTSGAESCSNSDDGTCCFEAPGGLL	Watanabe et al. (1995)
Dictyophora indusiata	GQPRQPQPQLLV	Wang and Ng (2003b)
Pleurotus eryngii	GEVVQYYP	Ng and Wang (2004)
Pleurotus sajor-caju	DNGEAGRAAR	Ngai and Ng (2004)
Ganoderma lucidum	HLPBVPSFAYGSIKVYIN	Wang et al. (2004)
Russulus virescens	TDHTLDTMMTHTLRD	Wang and Ng (2003c)
Pleurotus ostreatus	ETGVRSCNCAGRSFTGTDVTNAIRSARAGGSGN	Nomura et al. (1994)
Pleurotus tuber-regium	ALTAQDNRVRVGNRIVGNNFNFAAVQAAYY	Wang and Ng (2001)

[a] Full sequence available in the literature only for RNases from the mushrooms *Irpex lacteus*, *Lentinus edodes*, and *Pleurotus ostreatus*. Substantial homology exists only between RNases from *Irpex lacteus* and *Lentinus edodes*. The remaining RNases in this table do not show homology to any part of the sequences in RNases from *Irpex lacteus*, *Lentinus edodes*, and *Pleurotus ostreatus*

tuber-regium (Wang and Ng 2001) and *Thelephora ganbajun* (Wang and Ng 2004b), in that it is adsorbed on DEAE cellulose and Q Sepharose.

A comparison of the N-terminal ribonucleases of mushroom RNase (Table 2) reveals that mushroom RNases, with the exception of RNase from *Irpex lacteus* and *Lentinus edodes*, do not exhibit significant sequence homology to each other or to RNase from other organisms, such as mammals, plants, and bacteria. A similar picture is seen in the case of mushroom antifungal proteins (Ng 2004).

The molecular mass of *L. shimeiji* mushroom RNase (14.5 kDa) is larger than that of *Pleurotus ostreatus* RNase (11 kDa; Nomura et al. 1994). It is similar to those of *P. sajor-caju* RNase (12 kDa; Ngai and Ng 2004), *Pleurotus pulmonarius* (Ye and Ng 2002a), *Agaricus bisporus* RNase (14 kDa; Wang and Ng 2006), and *Pleurotus eryngii* RNase (16 kDa; Ng and Wang 2004). It is smaller than those of *Russulus virescens* RNase (28 kDa; Wang and Ng 2003c), *P. tuber-regium* RNase (29 kDa; Wang and Ng 2001), *T. ganbajun* RNase (30 kDa; Wang and Ng 2004b), and straw mushroom RNase (42 kDa; Wang and Ng 1999).

Pleurotus pulmonarius RNase is poly (C)-specific (Ye and Ng 2002a). RNases from *R. virescens* (Wang and Ng 2003c) and *Termitomyces globulus* (Wang and Ng 2003a) manifest co-specificity for poly (A) and poly (C). Portabella mushroom (*Agaricus bisporus*) RNase exhibits ribonucleolytic activity toward poly (A), poly (C), and poly (U) (Wang and Ng 2006). *Lyophyllum shimeiji* RNase is specific for poly (U) and poly (C), with much higher activity toward poly (U). *Pleurotus sajor-caju* RNase is poly (U)-specific (Ngai and Ng 2004). On the other hand, *P. tuber-*

regium (Wang and Ng 2001) and *P. ostreatus* (Nomura et al. 1994) RNases are specific for poly (G).

Lyophyllum shimeiji RNase is markedly more thermostable than *Thelephora ganbajun* RNase. In the latter, activity is much attenuated at 80°C compared with 20°C and is indiscernible at 100°C (Wang and Ng 2004b). On the other hand, the activity of *L. shimeiji* RNase at 80 and 100°C is higher than that observed at 20°C, but activity at 100°C is more or less the same as that observed at 20°C. Thus, *L. shimeiji* RNase would lose its RNase activity when the mushroom is eaten after it has been boiled, but retain some activity after treatment at 80°C.

The optimum pH for *L. shimeiji* RNase (pH 6.0) is similar to that for *P. tuber-regium* (Wang and Ng 2001) and *P. eryngii* (Ng and Wang 2004) RNases (pH 6.5). It is different from the pH 4.5 for portabella mushroom RNase (Wang and Ng 2006) and *R. virescens* RNase (Wang and Ng 2003c), and pH 8.0 for *P. pulmonarius* RNase (Ye and Ng 2002a) and *P. ostreatus* RNase (Nomura et al. 1994).

Ribonucleases from several types of ginseng (Lam and Ng 2001b; Ng and Wang 2001; Wang and Ng 2000) show antifungal activity, but *L. shimeiji* ribonuclease lacks similar activity. Many other mushroom ribonucleases (Wang and Ng 1999, 2003a, b, c, 2004a, b; Ye and Ng 2002a), except *P. sajor-caju* RNase (Ngai and Ng 2004), are also without antifungal activity. Bullfrog oocyte RNase (Liao 1992), but not *L. shimeiji* RNase, has lectin activity.

Lyophyllum shimeiji RNase is a new protein as suggested by its novel N-terminal sequence. It has some novel characteristics, including activity at high temperatures, a pH optimum and base specificity different from some of the isolated mushroom RNases, antiproliferative activity toward tumor cells and anti-HIV-1 reverse transcriptase activity, which have not been demonstrated for the majority of the isolated mushroom RNases.

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