Isolation and Characterization of a Type 1 Ribosome-Inactivating Protein from Fruiting Bodies of the Edible Mushroom (*Volvariella volvacea*)

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A novel single-chained ribosome-inactivating protein (RIP) with a molecular weight of ∼29 000 was purified from fruiting bodies of the edible mushroom *Volvariella volvacea* with a procedure involving ammonium sulfate precipitation, ion-exchange chromatography on DEAE-cellulose, and gel filtration on Superdex 75. The mushroom RIP, designated volvarin, exhibited a potent inhibitory action on protein synthesis in the rabbit reticulocyte lysate system with an IC_{50} value of 0.5 nM. Like most plant RIPs, volvarin acted as an *N*-glycosidase that depurinated rRNA from rabbit reticulocyte lysate, releasing a characteristic RNA fragment after treatment with aniline. It also exerted a deoxyribonuclease activity on supercoiled SV-40 DNA and demonstrated a strong abortifacient effect in mice.

Keywords: *Ribosome-inactivating protein; N-glycosidase; mushroom; Volvariella volvacea*

INTRODUCTION

Ribosome-inactivating proteins (RIPs) are a family of plant proteins that exhibit several interesting biological properties (Barbieri et al., 1993; Girbes et al., 1996). They damage eukaryotic ribosomes via their rRNA *N*-glycosidase activity, which depurinates ribosomes at a specific nucleotide, A-4324, of the 28S rRNA. This site of nucleotide is highly conserved and essential for ribosomal function (Endo et al., 1987). RIPs from most plants generally possess antiviral activities; however, some RIPs, such as those obtained from cereals barley and maize, show high antifungal activities (Barbieri et al., 1993; Seetharaman et al., 1996). There are two types of RIPs: Type 1 RIPs are single-chained proteins that strongly inhibit protein synthesis in a cell-free system. Type 2 RIPs are proteins consisting of two subunits (a type 1 RIP A-chain and a lectin B-chain) connected by a disulfide bond (Barbieri et al., 1993). Type 1 RIPs are more common and have been identified and purified from >40 plants. The first type 1 RIP identified was pokeweed antiviral protein (Irvin, 1983). One of the most interesting type 1 RIPs is trichosanthin, which is obtained from the tubers of Chinese medicinal herb *Trichosanthes kirilowii* (Zhang and Wang, 1986; Ng et al., 1992). Trichosanthin selectively inhibits the replication of human immunodeficiency virus (HIV) in both lymphoid and mononuclear phagocyte cells in vitro and in monocytes/macrophages isolated from HIV-infected individuals in vivo (McGrath et al., 1989). Phase I and II clinical trials on trichosanthin were carried out in the United States (Byers et al., 1990; Kahn et al., 1992). However, adverse neurological toxicities were observed in some patients (Pulliam et al., 1991). Thus, it would be worthwhile to look for an antiviral RIP with fewer side effects.

There are several studies on mushroom bioactive proteins. The cardiotoxic protein volvatoxin, which has a molecular weight ∼25 000, has been isolated from the edible mushroom *Volvariella volvacea* (Lin et al., 1973). However, its physicochemical characteristics and biological functions suggest that it does not bear a relationship to RIPs. An antiviral protein (MW 23 000) has been purified from the fruiting bodies of *Lentinus edodes*. It is an acidic protein but there is no direct evidence that it is an RIP (Kobayashi et al., 1987). Bolesatine, a toxic protein isolated from the mushroom *Boletus satanas* Lenz, inhibits protein synthesis in vitro with an IC_{50} value of 0.14 μM. Its MW is 63 000, suggesting that it may be a type II RIP, but Licastro et al. (1993) emphasized that it was a toxic lectin because it had strong mitogenic activity and immunologic stimulus for the release of IL-1 and IL-2 at low concentrations (0.01−0.1 μg/mL). In this paper, we report for the first time the presence of RIP in a popular edible mushroom, *V. volvacea*. Its MW is ∼29 000, and the median concentration (IC_{50} value) of its protein synthesis inhibitory activity is 0.5 nM. We designate it volvarin. It exhibits rRNA *N*-glycosidase, deoxyribonuclease, and abortifacient activities.

MATERIALS AND METHODS

Purification Procedure. Fresh fruiting bodies of the cultivated mushroom *V. volvacea* obtained from the mushroom house of The Chinese University of Hong Kong were homogenized in 0.2 M NaCl, and the homogenate was then extracted overnight with 0.2 M NaCl at 0−4 °C. Following centrifugation at 22100*g* for 12 min, the supernatant was collected and

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$(NH_4)_2SO_4$ was added to 30−80% saturation. Salt was removed by dialysis against several changes of distilled water and finally against 0.01 M phosphate buffer (pH 6.5). DEAE-cellulose that had been pre-equilibrated with the same buffer was then added to adsorb colored material. The mixture was stirred for 30 min. After filtration, the filtrate was lyophilized. The resulting powder was dissolved in and dialyzed against 0.01 M Tris−acetate buffer (pH 7.1) and then applied to a DEAE column (1 × 30 cm) pre-equilibrated in the same buffer. After appearance of the breakthrough peak, adsorbed materials were eluted with 0.025 M Tris−acetate buffer (pH 7.1) at a flow rate of 40 mL/h. The first major peak exhibiting ribosome-inactivating activity was collected. Ultrafiltration using a membrane with a MW 10 000 cutoff was carried out and followed by gel filtration on a Superdex 75 column (1.5 × 80 cm). The fractions were collected and tested for ribosome-inactivating activity.

Estimation of Protein Molecular Weight and Purity. The concentration of protein was determined colorimetrically with the method of Lowry et al. (1951). Molecular weight and purity of proteins were determined by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE), using 12.5% acrylamide running gel and 5% stacking gel. The gel was stained with 0.1% Coomassie brilliant blue R-250 and then destained with 7% acetic acid. The MW standards (Pharmacia, Uppsala, Sweden) used included phosphorylase (MW 94 000), bovine serum albumin (MW 67 000), ovalbumin (MW 43 000), carbonic anhydrase (MW 30 000), and soybean trypsin inhibitor (MW 21 000).

Assay for Cell-Free Protein Synthesis Inhibitory Activity. The mushroom protein obtained from the aforementioned procedure was tested for the ability to inhibit incorporation of [^{35}S]methionine into protein (Pelham and Jackson, 1976). Four microliters of sample (in 0.5 mg/mL BSA) and 6 μL of a mixture including [^{35}S]methionine, hemin, creatine kinase, creatine phosphate, KCl, and $MgCl_2$ were added to 30 μL of untreated rabbit reticulocyte lysate (Promega, Madison, WI), and incubated for 30 min at 37 °C. A 5-μL aliquot of the reaction mixture was added to 1 mL of distilled water to stop the reaction, and this was followed by addition of 0.5 mL of 10% H_2O_2 in 1 N NaOH and incubation at 30 °C for 30 min. Proteins were precipitated with 3 mL of 25% trichloroacetic acid (TCA) containing 2% casein hydrolysate at 4 °C and collected by filtration through a Whatman GF/B filter paper using a vacuum pump. The filter was washed with 8% TCA and acetone, and the radioactivity of [^{35}S]methionine incorporated into protein was measured by liquid scintillation counting.

Assay for rRNA *N*-Glycosidase Activity. Untreated rabbit reticulocyte lysate (Promega) was incubated with volvarin in Tris-HCl buffer (25 mM, pH 7.6) for 30 min at 37 °C. The reaction was terminated by addition of sodium dodecyl sulfate to a final concentration of 0.5%. The rRNA was then extracted with phenol and precipitated with ethanol. To cleave the "aniline-labile site", RNA was dissolved in 1.0 M aniline−acetate (pH 4.5) and incubated for 3 min at 65 °C. After precipitation by ethanol, RNA was obtained and electrophoresed on 1.2% agarose gel containing 50% formamide at 50 V for 1.5 h. The gel was stained with ethidium bromide (Morris and Wool, 1992). The appearance of an Endo's band on electrophoresed gel as a result of the release of a new fragment of 400−500 nucleotides following a treatment with acidic aniline is used as a specific indicator of *N*-glycosidase activity (Endo et al., 1987, 1988).

Assay for Deoxyribonucleolytic Activity. To test for the deoxyribonucleolytic activity, supercoiled double-stranded SV-40 DNA (200 ng; Bethesda Research Laboratories) was incubated with various amounts of volvarin (10−800 ng) in sodium acetate buffer (10 mM CH_3COONa buffer, pH 5.5, containing 2 mM $MgCl_2$ and 2 mM $CaCl_2$) at 37 °C for 60 min. The reaction was terminated by adding loading buffer (60 mM EDTA, 0.25% bromophenol blue, and 30% glycerol). Electrophoresis was performed in 1% agarose with standard TBE buffer (89 mM Tris−borate, 89 mM boric acid, and 2 mM EDTA, pH 8.0) at a constant voltage of 60 V. The gel was stained for DNA with ethidium bromide at a final concentration of 0.2 μg/mL for 45 min and then destained in water. The DNA bands were visualized on a UV transilluminator and photographed with a Polaroid MP_4 camera.

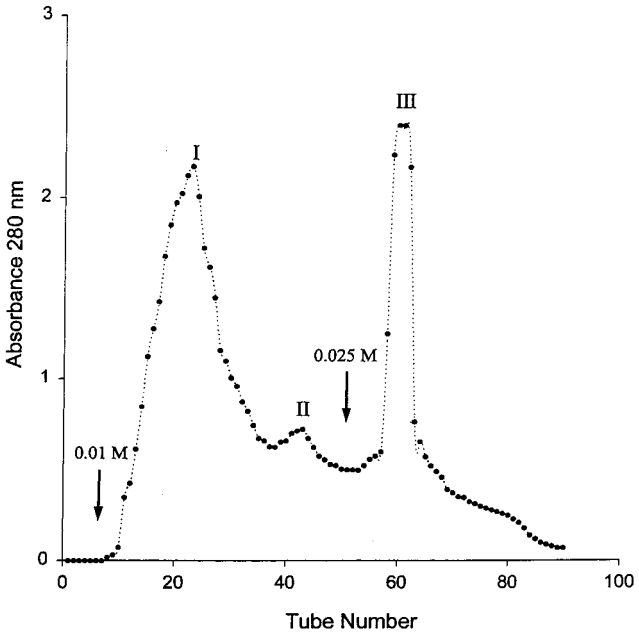

Figure 1. Ion exchange chromatography on DEAE-cellulose column. Fresh fruiting bodies of *V. volvacea* were extracted with 0.2 M NaCl, followed by precipitation with ammonium sulfate (30−80% saturation) and decolorization with DEAE-cellulose. The fraction unadsorbed on DEAE-cellulose was dialyzed against 0.01 M Tris−acetate buffer and then applied on a DEAE-cellulose column (1 × 30 cm) that had been pre-equilibrated with the same buffer. The column was eluted stepwise with Tris-acetate buffer (0.01 M and then 0.025 M, pH 7.1) at a flow rate of 40 mL/h.

Assay for Abortifacient Activity. Pregnant ICR mice obtained from the animal house of The Chinese University of Hong Kong were divided into two groups. On the tenth day of gestation, five mice used for the experimental treatment group were each given a single dose of volvarin (0.15 mg dissolved in 0.3 mL of physiological saline) by intraperitoneal injection. Another three mice that received only physiological saline were used as the control group. Five days after volvarin administration, the mice were killed by cervical dislocation. The uteri were cut open, and the number of fetuses and their condition were noted (Ooi et al., 1995). When the number of dead or resorbed fetuses found in the two uterine horns was >50% of the total implantation sites, pregnant mice were considered to have undergone abortion.

RESULTS AND DISCUSSION

The present study demonstrates the existence of a RIP with rRNA *N*-glycosidase activity in fruiting bodies of the mushroom *V. volvacea*.

The fruiting bodies of *V. volvacea* contain a vast amount of polysaccharides and fairly active oxidation enzymes such as laccase and polyphenol oxidase. To remove these materials and to decolorize the extract, an adsorption step using DEAE-cellulose at pH 6.5 was performed prior to column chromatography. It was found that the fraction with ribosome-inactivating activity was loosely bound to the DEAE-cellulose column. A major peak with ribosome-inactivating activity was obtained when the ionic concentration was increased from 0.01 to 0.025 M (Figure 1). This peak was concentrated and then applied on a Superdex 75 column. The resulting peak 3 (Figure 2) possessed strong ribo-

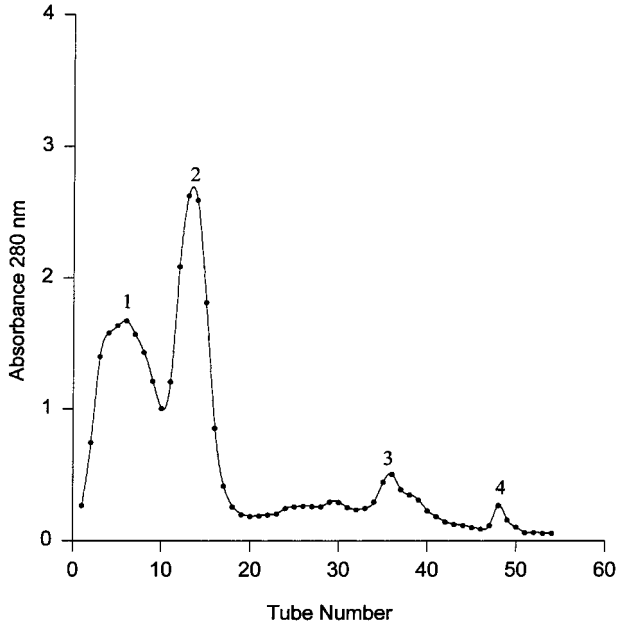

Figure 2. Gel filtration chromatography on Superdex 75 column. Peak III was obtained from the DEAE-cellulose column after concentration was applied on a Superdex 75 column (1.5 × 80 cm) that was eluted with 0.025 M Tris−acetate buffer (pH 7.1) at a flow rate of 32 mL/h.

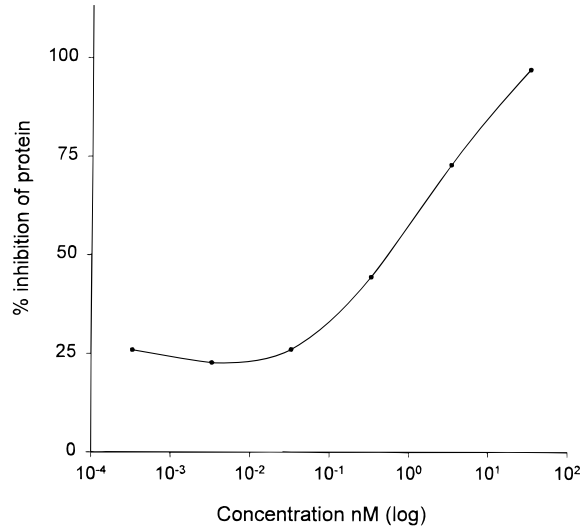

Figure 3. Inhibition of cell-free protein synthesis by volvarin. The in vitro translation reaction mixture contained [^{35}S]-methionine, rabbit reticulocyte lysate, and volvarin. Protein with incorporated radioactive methionine was precipitated with TCA, and radioactivity was measured using a liquid scintillation counter. Incorporation of radioactive methionine in the absence of RIP (control) was regarded as 100% of protein synthesis activity.

some-inactivating activity. It inhibited incorporation of [^{35}S]methionine into protein in the rabbit reticulocyte system. The median inhibitory concentration (IC_{50}) was determined to be 0.5 nM (Figure 3). This RIP obtained from *V. volvocea* was designated volvarin. Its homogeneity was confirmed using SDS−PAGE (Figure 4). The result of SDS−PAGE showed that volvarin was a single-chained protein with a MW of ∼29 000. This value is comparable to those for many type 1 RIPs derived from plants (Barbieri et al., 1993).

Our results also suggested that volvarin, like many other type 1 RIPs (Barbeiri et al., 1993), exhibited abortifacient activity because all 54 fetuses found in the

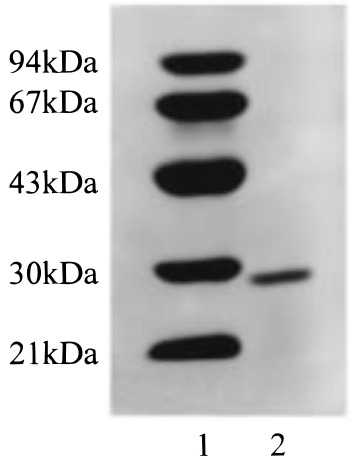

Figure 4. SDS−PAGE of volvarin. The fraction having ribosome-inactivating activity (volvarin) was subjected to SDS−PAGE using 12.5% acrylamide running gel and 0.4% SDS (pH 8.3). The gel was stained with 0.1% Coomassie blue R-250 and then destained with 7% acetic acid: lane 1, MW markers (MW 96 000, 67 000, 43 000, 30 000, and 21 000 purchased from Pharmacia); lane 2, mushroom RIP volvarin.

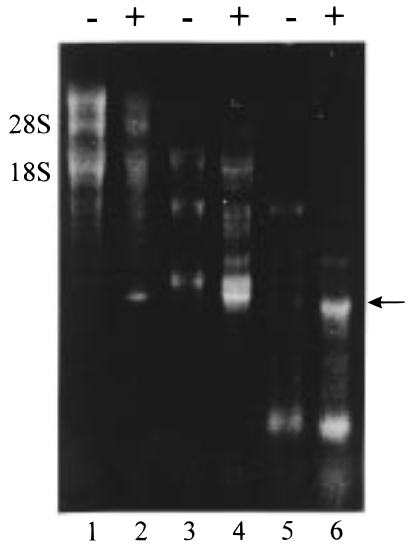

Figure 5. Effect of volvarin on intact rRNA (*N*-glycosidase activity of volvarin). Untreated rabbit reticulocyte lysates were incubated with volvarin at various concentrations (0.3 nM for lanes 1 and 2; 3.0 nM for lanes 3 and 4; 30 nM for lanes 5 and 6) in Tris-HCl buffer for 30 min at 37 °C and terminated by addition of SDS (0.5%). The extracted rRNA was dissolved in 1 M aniline−acetate (pH 4.5) and incubated at 65 °C for 3 min. After precipitation with ethanol, RNA was analyzed by electrophoresis in 1.2% agarose gel containing 50% formamide at 50 V for 1.5 h. The gel was stained with ethidium bromide. For lanes 2, 4, and 6, rRNA was treated with acidic aniline. For lanes 1, 3, and 5, rRNA was not treated with aniline. The arrow denotes the presence of Endo's band, indicating a new fragment of ∼450 nucleotides produced as a result of acidic aniline treatment.

5 midterm pregnant mice treated with a single dose of volvarin were dead at the time of autopsy, and thus termination of all pregnancies of the treated mice was evident (Ooi et al., 1995).

Depurination of RNA due to the *N*-glycosidase activity of volvarin is shown in Figure 5. When ribosomes of rabbit reticulocyte lysate were incubated with volvarin, followed by a treatment of the extracted rRNA with aniline−acetate (pH 4.5), a new RNA fragment appeared on the gel, which is sometimes known as an Endo's band. Treatment without aniline had no evi-

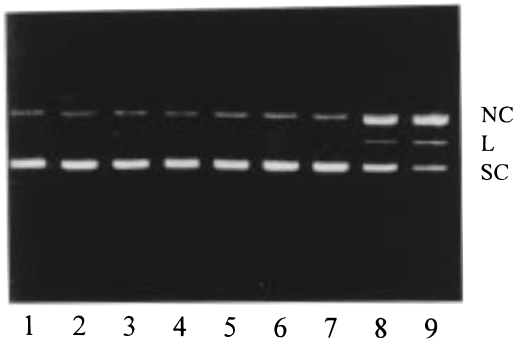

Figure 6. Deoxyribonucleolytic activity of volvarin. Supercoiled double-stranded SV-40 DNA (200 ng) was incubated with various amounts of volvarin in sodium acetate buffer (pH 5.5) at 37 °C for 1 h. Electrophoresis was performed in 1% agarose gel with standard TBE buffer at a constant voltage of 60 V. The gel was stained for DNA with ethidium bromide. Lane 1, control containing only SV-40 DNA; lanes 2−9, SV-40 DNA with, respectively, 10, 20, 50, 100, 200, 300, 500, and 800 ng of volvarin. NC, nicked circular; L, linear; SC, supercoiled forms of SV-40 DNA.

dence of Endo's band. Volvarin seemed to make a modification in rRNA, causing it to become susceptible to cleavage by aniline, and thus resulted in the production of a new fragment of ~450 nucleotides (Endo et al., 1987; Endo, 1988). Since the treatment of rRNA with RIPs renders the phosphodiester bond of RNA sensitive to cleavage by aniline, the properties of this "aniline-labile site" and the release of adenine are used as criteria in identifying an *N*-glycosidase (Endo et al., 1987). The results indicated that volvarin, like most plant RIPs, inhibited protein synthesis through its rRNA *N*-glycosidase activity (Barbeiri et al., 1993). However, its mechanism of action is different from that of α-sarcin, RIP of the mold *Aspergillus giganteus*, which inactivates ribosomes by its endonuclease activity which cleaves the phosphodiester bond between the G-4325 and A-4326 sites (Endo et al., 1982; Campos-Olivas et al., 1996).

Furthermore, volvarin exhibited a deoxyribonucleolytic activity (Figure 6). When 200 ng of supercoiled, double-stranded SV-40 DNA was used as a substrate, volvarin up to an amount of 400 ng could slightly transform the DNA to nicked circular form as indicated by the higher intensities with increasing concentrations. When larger amounts (500 or 800 ng) of volvarin were used, the nicked circular SV-40 DNA was more prominent and, in addition, new linear forms of the DNA also appeared on the gel (lanes 8 and 9 of Figure 6). Some ribonucleolytic activity of volvarin could also be observed at concentrations of 3 and 30 nM (Figure 5). In lane 3 of Figure 5, in which 3 nM of volvarin was used and the RNA was not treated with aniline, the 28S rRNA almost disappeared and a new band close to Endo's band appeared. In lane 5, in which 30 nM volvarin was used, both 28S and 18S RNA completely disappeared. Several new bands appeared indicating that they were not totally a smear. Furthermore, no matter how high a concentration of RIP was used, Endo's band could always be observed after treatment with aniline (see lanes 2, 4, and 6 in Figure 5). This means that the inherent *N*-glycosidase activity of volvarin could not be affected by ribonucleolytic activity. It is possible that the ribonucleolytic activity is an intrinsic character of volvarin. The deoxyribonuclease activity of some RIPs has been reported (Li et al., 1991; Go et al., 1992; Ling et al., 1995; Roncuzzi and Gasperi-Campani, 1996; Nicolas et al., 1997). Supercoiled HIV-LTR DNA treated with the RIP MAP30 is converted mainly to the relaxed form and only a trace amount of the linear form resulted (Lee-Huang et al., 1995). Roncuzzi and Gasperi-Campani (1996) demonstrated that single-chain RIPs dianthin 30, saporin 6, and gelonin exert a specific nuclease activity on DNA by introduction of highly selective cleavage into supercoiled DNA molecules of plasmids pBR322 and ϕX174. It is noteworthy that saporin, a well-known plant RIP, releases adenine not only from various RNAs but also extensively from DNA and poly(A) (Barbieri et al., 1997). It is likely that depurination of DNA may also have a role in antiviral activity, senescence, and pathogenesis (Huang et al., 1992; Bolognesi et al., 1996; Barbieri et al., 1997). Besides, saporin can cleave and liberate up to 33 molecules of adenine from 1 molecule of rat liver rRNA, indicating it is capable of depurinating rRNA at more than one site (Barbieri et al., 1992). Therefore, the class of RIPs has recently been assigned as polynucleotide:adenosine glycosidases (Barbieri et al., 1997). Furthermore, it was found that trichosanthin and some other RIPs can react with 5′-AMP and convert it to adenine and adenosine (Hao et al., 1995; Chen et al., 1996). From these studies, the reaction products of RIPs with 5′-AMP can be correlated with phosphoesterase activity (Chen et al., 1996). The above-mentioned recent observations indicate that RIPs might still have some unexpected activities and functions which await further investigations. This may provide a new clue for the study of the natural functions and potential biomedical applications of RIPs.

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