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Infectious RNA Viruses in the Edible Mushroom *Pleurotus* spp.

Jeong Soo Park and Young-Ho Kim*

Laboratory of Microbiology, Department of Biology, College of Natural Sciences, The University of Suwon, Suwon, Korea

**Department of Biology, College of Natural Sciences, The University of Suwon, Suwon 445-743, Korea*

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Double-stranded RNA(dsRNA) viruses and single-stranded RNA(ssRNA) viruses were detected in a strain of *Pleurotus* mushroom cultivated in a farm. Those fungal viruses were purified in the pH 6.0 or pH 7.2 using CsCl or Cs_2SO_4 buoyant density centrifugation. Each viral particles were not completely separated at any trials. However, mushroom bacilli-form virus contains a single major nucleic acid with 0.7 Kb ssRNA, which might code for 20 Kd viral capsid protein. The dsRNAs are encapsidated into spherical-form viruses, whereas ssRNA viral genomes are encapsidated into two different sizes of bacilli-form particles. A healthy-looking mushroom also contained some spherical-form viruses with dsRNAs. Laboratory strains of *Pleurotus ostreatus* and a cultivated strain of *P. sajor-caju* did not show any viral particles. Mushrooms with specific disease symptoms, however, contained at least four different sizes of spherical-form viruses. Thus, we concluded that a bacilli-form virus cause a severe disease symptoms of abnormal growth on mushroom development.

Key words: ***Pleurotus florida*, *Pleurotus ostreatus*, bacilli-form virus, oyster mushroom**

The virus-like particles isolated from diverse fungi have been found to have many similar physical and chemical properties, whereas most particles were not shown as infectious features. Those fungal viruses have been characteristically contained multipartite double-stranded RNA(dsRNA) genomes which are generally encapsidated as spherical nucleoprotein particles (5, 10, 18, 21, 24). Different size and density of their particles give rise to the appearance of multiple centrifugal and density of their component (6, 34). In most of the fungal viruses, the development of virus has no effect on the cellular growth or development of the fungi. In general, fungal viruses are known to be avirulent or latent and cause no obvious phenotypic changes in the host fungi (6, 21).

La France disease (die-back) of the cultivated white button mushroom *Agaricus bisporus*, however, was reported as a first experimental evidence of the mushroom disease (17). Some other viruses cause lytic plaque formation in fungi such as *Penicillium chrysogenum* (4), *Schizophyllum commune* (20), *Saccharomyces cerevisiae* (3), *Ustilago maydis* (6), human pathogenic fungus *Blastomyces dermatitidis* (19). The virus was also found to be associated with a severe disease of the cultivated white-button mushroom. The primary symptom of the infectious disease was shown as a die-back of mycelium resulting in a loss of the crop (32). Several evidence for the existence of a disease-specific pattern of dsRNA were provided from diseased mushroom but not from an apparently healthy-looking mushroom (15, 16, 17, 22, 33). Most of these viruses showed a spherical morphology encapsidated with multipartite dsRNA genomes.

A bacilli-form virus from a diseased mushroom, *Agaricus bisporus*, has been first extensively purified and shown to be a unique mycovirus in that the genome is single-stranded RNA(ssRNA) (2, 28). They proposed that the bacilli-form virus was the first mycovirus shown to possess a ssRNA genome but contaminated with the 25- and 35-nm spherical viruses. Six morphologically distinct virus-like particles were most frequently identified in the unhealthy-looking mushroom, *Agaricus bisporus* (7, 17, 25, 26). Any apparently healthy-looking mushrooms, however, showed a dectectable amounts of dsRNAs (15).

* To whom correspondence should be addressed.

although Wach *et al* (1987) could not detect any dsRNAs in the healthy-looking mushrooms. Five of the six different virus-particles were spherical particles with diameters of 19-, 25-, 29-, 34-35-, and 50-nm. Another unique bacilli-form particle measuring 19×50-nm, was clearly shown to be contained a ssRNA genome which caused a severe disease symptom in the cultivated mushrooms.

Recently, it was reported that another mushroom, oyster mushroom, was also infected with spherical virus-like particles (12). Two different oyster mushroom, *Pleurotus florida* and *P. ostreatus*, infected with viruses showed a slow growth on sawdust and rice straw substrates (13). They observed that the pinhead was formed too densely or too rarely sometimes and the stipes of the mushrooms were longer and slightly bent with smaller cap. The mushroom infected with viruses formed many branches on their stipes. They purified only two different sizes of spherical-form viruses from unhealthy-looking mushrooms. It was found that abnormally grown *Pleurotus* species showed a typical symptoms of disease due to dsRNA virus infections (14). They could not isolate any detectable dsRNAs from healthy-looking mushroom isolates.

During our studies on the development of the oyster mushroom from several *Pleurotus* isolates and laboratory strains, we isolated a bacilli-form virus as well as several different sizes of spherical-form viruses.

We also conclusively demonstrated that an unique bacilli-form virus encapsidated a ssRNA genome similar to that of *Agaricus bisporus*. Furthermore, we suggest that this infectious RNA mycovirus might cause a severe retardation of mushroom development.

Materials and Methods

Mushroom strains and cultures

Three different groups of oyster mushroom, *Pleurotus ostreatus*, have been selected through this study; (1) healthy-looking oyster mushrooms, *Pleurotus* spp., (2) unhealthy-looking mushroom developed as an abnormal growth, (3) mycelial mats of several different laboratory strains of *P. ostreatus* designated as NFFA2, NFFA 2m1, NFFA 4001 and NFFA 4501 distributed from the department of microbiology, Seoul National University. These strains were inoculated into the malt extract medium to cultivate their mycelium. Liquid cultures of mycelium were collected and used for the purification of virus particles. Healthy-looking mushrooms were also collected from mushroom farms or market.

Purification of viruses

To purify the fungal virus-like particles, we used the procedures followed by methods of Harmsen *et al.*, (1989) with some modifications.

About 200 grams of diseased or healthy mushrooms (mycelia) were harvested and then washed with 50 mM sterilized phosphate buffer (pH 7.2 or changeable) followed by addition of 200 ml same buffer containing 0.5% 2-mercaptoethanol. The mycelia were broken by grinding in a homogenizer for 5 to 10 min with intervals at 4C. The cell debris was removed by low-speed centrifugation at 8,000 rpm for 20 min in a Sorvall centrifuge and the pellet was rehomogenized as described above. The virus-like particles were either directly collected by ultracentrifugation in the presence of 20% sucrose cushion or precipitated by addition of 6% polyethylene glycol (MW 8,000) in the presence of 0.15 M NaCl. The virus pellet was resuspended with same phosphate buffer and clarified. The virus particles were subjected to 10~50% sucrose density gradient. The density gradient centrifugation was carried out at 24,000 rpm for 3.5 hrs by using a Sorvall SW28 rotor (Sorvall Inc.). Specific light scattering band produced by virus preparation in sucrose density gradient centrifugation was collected by high-speed centrifugation at 40,000 rpm for 2 hrs at 4C in a Sorvall TFT 50.38 rotor. The virus pellet was resuspended in the phosphate buffer and stored at −70C. For further purification of particles, partially purified virus samples were applied onto CsCl or Cs_2SO_4 buoyant density gradient centrifugation prepared in phosphate buffer and centrifuged for 24 hrs to be equilibrium (120,000×*g*, 18 hrs at 4C) in a Sorvall AH 627 rotor. The viral particles (VPs) were then fractionated and collected.

Analysis and characterization of *Pleurotus* virus-like particles

(i) Observation of Particles by Electron Microscope

Virus preparations were negatively stained with 0.1% uranylacetate (pH 6.0) for 20 sec and then examined with a Model H-800 Hitachi electron microscope.

(ii) Nucleic Acid Analysis

Isolation of the viral nucleic acid was done by the single-phase phenol-SDS method of Diener and Schneider (1968). Nucleic acids were precipitated with cold ethanol and resuspended with minimal volume of TE buffer (pH 8.0). Aliquots of viral nucleic acid were made with 2× SSC buffer (1× SSC buffer, pH 7.2, contains 0.15 M NaCl and 0.015 M Na-citrate). Pancreatic ribonuclease A (RNase A) or DNase I was serially diluted and added to one aliquots, respectively whereas the second aliquot with distilled water served as control. Each samples were incubated at room temperature for 30 min prior to gel electrophoresis. Nucleic acids of virus were analyzed on 1.0% agarose gel electrophoresis.

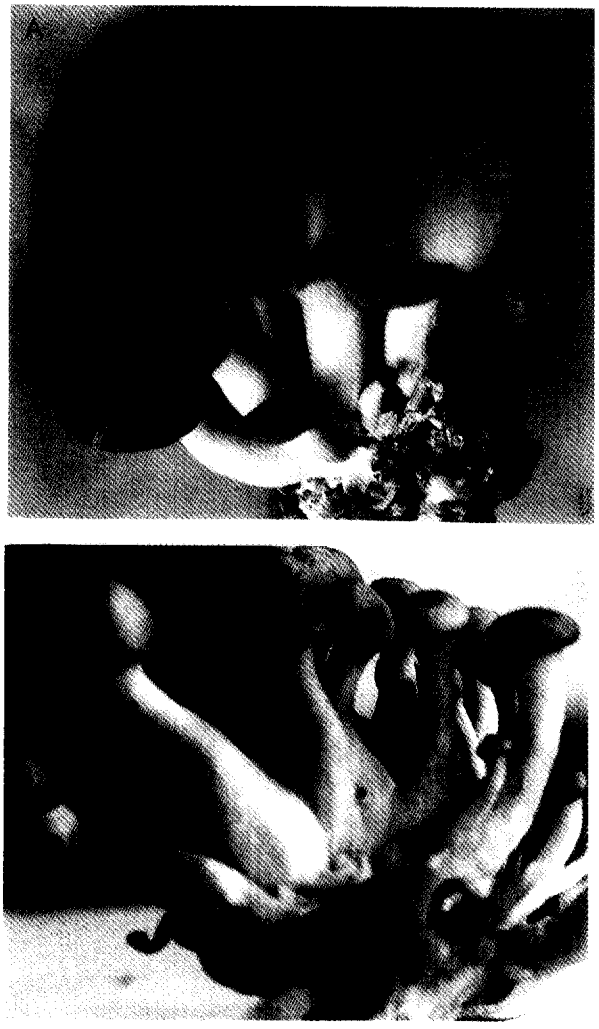

Fig. 1. Comparison of fruiting bodies of mushrooms between healthy-looking and diseased or unhealthy-looking infected with viruses. (A) A normally grown mushroom without any disease symptoms had a larger cap with dark grey color. (B) Fruiting bodies of diseased mushrooms infected with RNA viruses showed a long stipe with small cap or branched stipes.

(iii) Viral Proteins

The virion-associated polypeptides were resolved by SDS-polyacrylamide gel electrophoresis using the discontinuous buffer system. Viral particles in electrophoresis loading buffer (1% SDS, 10% glycerol, 10 mM Tris-HCl, pH 7.6, 0.005% bromophenol blue, 1% 2-mercaptoethanol) were denatured by heating for 2-3 min at 90C just prior to electrophoresis. Following electrophoresis, polypeptides were stained with 0.2% Coomassie brilliant blue G-250. Gels were destained in the same solvent with a methanol : water : glacial acetic acid (5 : 5 : 1, v/v) at room temperature until the background stain was removed.

Results

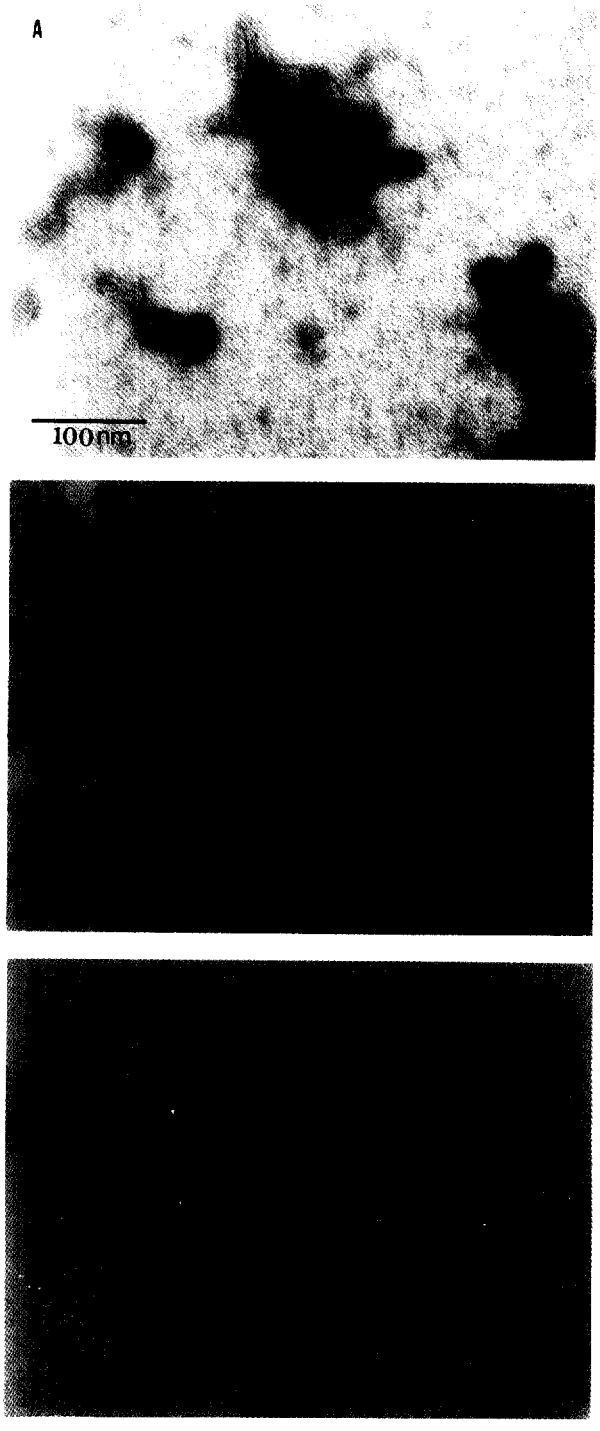

Fig. 2. Electron Micrographs of virus particles isolated from cultivated *Pleurotus florida*. Purified virus-like particles were negatively stained with uranyl acetate (×30,000): (A) Some spherical-form and bacilli-form viruses were co-infected into a diseased mushroom, (B) only spherical-form virus were infected into unhealthy-looking mushroom, and (C) some other spherical-form viruses were purified from healthy-looking mushroom.

Purification of viruses

Virus-like particles were partially purified and analyzed from a disease-specific, healthy-looking and labora-

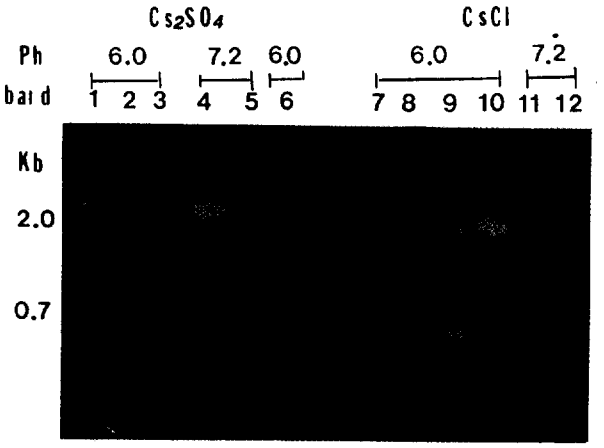

Fig. 3. Analysis of RNA of viruses purified from cultivated *Pleurotus florida* mushroom. RNA samples were electrophoresed in 1% agarose gel and stained with ethidium bromide. All RNAs were purified from viral particles infected into diseased or unhealthy-looking mushrooms, except lane 6 from healthy-looking mushroom. Each lanes represented the bands after CsCl or Cs_2SO_4 equilibrium ultracentrifugation in the two different buffers of pH values.

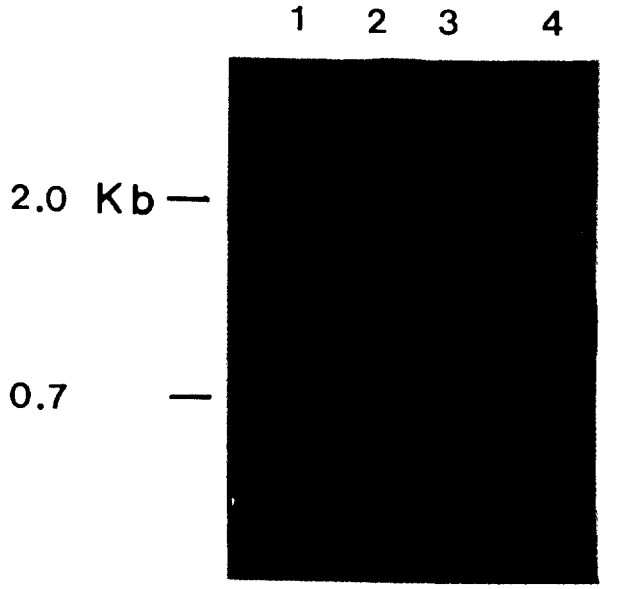

Fig. 4. Pancreatic RNase A and DNase I sensitivity assay of viral RNA isolated from diseased mushroom. Lane 1, RNA only; lane 2, RNA treated with RNase A (10 mg/ml) in 6× SSC buffer; lane 3, RNA treated with RNase A (0.01 mg/ml) in 6× SSC buffer; lane 4, RNA treated with DNase I.

tory strains of *Pleurotus* mushroom, respectively. Diseased or healthy-looking fruiting bodies of mushrooms were obtained from mushroom farms in the near area of Suwon. Diseased or unhealthy-looking mushrooms infected with specific viruses produced a bent, thin and long stip with small darker brownish cap, compared to the healthy-looking and normal mushroom (Fig. 1). Negatively stained virus particles purified from those mushrooms were analyzed by electron microscopy. Slow and abnormal growth of oyster mushroom was infected with spherical virus particles. As shown in Fig. 2A, abnormally grown *Pleurotus florida* in the mushroom farm was severely diseased and infected with spherical-form and/or bacilli-form viruses, whereas laboratory strains of *Pleurotus* mushroom did not contain any virus-like particles. Four different sizes of spherical-form viruses were estimated as diameters of 21-47 nm and shown as an infectious agents even in the healthy-looking specimens (Fig. 2B and 2C). Bacilli-form viruses were not found in the healthy-looking mushrooms and in the laboratory strains of *Pleurotus* spp.. The sizes of these bacilli-form viruses were calculated as 16×47 nm and 4×23 nm, respectively, but smaller one was not evenly found. The majority and larger size of bacilli-form viruses was not easily separated from spherical-form viruses.

Analysis of viral RNAs

All viral particles isolated from healthy or unhealthy-looking mushrooms were used to detect viral nucleic acids. Each nucleic acids were extracted from each fractions of the CsCl or Cs_2SO_4 gradient, separated in an 1.0% agarose gel and stained with ethidium bromide (Fig. 3).

When the viruses were purified from a diseased mushroom in the Cs_2SO_4 density gradient centrifugation fractions, dsRNA patterns at pH 7.2 (Fig. 3, lanes 4 and 5) were more clearer than those at pH 6.0 (Fig. 3, lanes 1, 2, and 3). It was also found that healthy-looking mushrooms were infected with spherical-form viruses containing dsRNAs only (Fig. 2C and Fig. 3, lane 6). Healthy-looking mushroom showed some infection of spherical-form viruses with 2.0~2.3 Kbp length of dsRNA (Fig. 3, lane 6).

When the viruses, however, were fractionated from diseased mushroom in the CsCl density gradient centrifugation fractions, viral RNAs including ssRNA at the acidic conditions were clearly revealed on the agarose gel (Fig. 3, lanes 7 to 12). Only 2.0~2.3 Kbp length of RNAs shown on the lanes 5, 7 and 8 of Figure 3 was extracted from a spherical-form viral particle. Small RNA of 0.7 Kbp length as well as 2.0 Kbp length of RNA was found in the virus sample which contained the bacilli-form virus (Fig. 2A). This 0.7 Kbp length of RNA was identified as ssRNA by the RNase A sensitivity test (Fig. 4). That is, 0.7 Kbp size of RNA was sensitively digested by RNase A, but 2.0 Kbp or slightly different sizes of RNA were not sensitive to RNase A or DNase I.

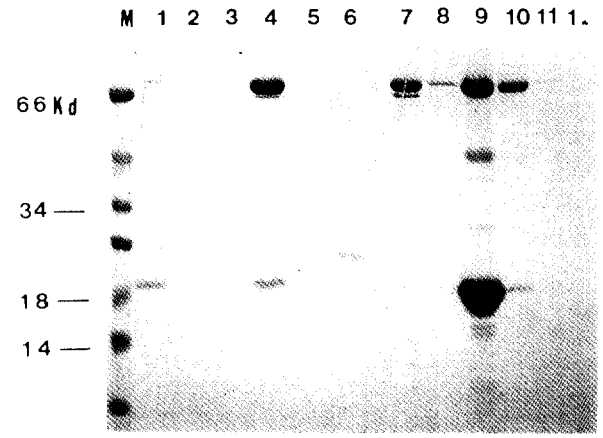

Fig. 5. Analysis of structural proteins of VLP isolated from a diseased or healthy-looking mushrooms. All viral proteins were extracted from the viruses of the samples shown in Fig. 3. 10% SDS-polyacrylamide gel electrophoresis was followed by fixation and stained with Commassie blue. Each lanes of 1 to 12 represent the same lanes of Fig. 3, respectively.

Viral capsid proteins

Analysis of the virion-associated proteins by 10% SDS-PAGE revealed polypeptides which was presumed to be the viral capsid proteins (Fig. 5). A normally grown mushroom contained a single spherical-form virus. This virus was assumed to be encapsidated with a single polypeptide of a molecular weight of 22 Kd, which was presumed to be the capsid protein (Fig. 5, lane 6). Abnormally grown mushroom was, however, found that several different forms of viruses were isolated and showed a complex protein patterns (Figs. 3 and 5). These results were presumed that 2.0 Kbp length of dsRNA of spherical-form virus was encapsidated with 70 Kd protein, whereas 0.7 Kbp length of ssRNA of bacilli-form virus was encapsidated with 20 Kd proteins.

Discussions

Results shown in the Figures 1 and 2 suggest that oyster mushrooms are infected with isometric virus particles and unique bacilli-form virus particles and then abnormally developed with slow and abnormal growth. Healthy-looking mushrooms are infected with isometric viruses only. The disease symptoms of mushroom infected with these bacilli-form and spherical-form viruses were more severe than those of the healthy-looking mushroom infected with spherical-form viruses only. It is the first time to find out the presence of bacilli-form virus particles from *Pleurotus* mushroom, which might be related with mushroom disease. Other higher fungi such as common mushroom *Agaricus bisporus* (1, 8), oak mushroom, *Lentinus edodes* (23, 29, 30, 31), also were infected with several different isometric virus particles and bacilli-form virus particles. Tavantzis *et al.* (1980) also purified bacilli-form virus from diseased *Agaricus bisporus*. In case of healthy high-yielding *Agaricus bisporus* mushrooms, any dsRNAs were not found to be infected. In addition, virus particles did not occur regularly in apparently healthy mushrooms. We have now, however, confirmed that spherical virus particles were identified to be infected into healthy-looking mushrooms, whereas co-infection of bacilli-form virus with spherical-form viruses caused a severer disease or symptomatic development.

When a purification procedure was applied on the second Cs_2SO_4 centrifugation after CsCl centrifugation, bacilli-form viruses were not separated from spherical-form viruses. Compared to the viral RNAs infected into healthy-looking mushroom, unhealthy-looking mushrooms were infected with some other spherical-form viruses containing dsRNAs and also with bacilli-form viruses containing ssRNA. These facts shows that the relationship between the presence of the ssRNA and the developmental expression of the disease is apparently evident and complex. The presence of the dsRNAs in the diseased mushroom does not appear necessarily to lead to malformed fruiting bodies but it might lead to the way of going to be malformed development. Smaller sizes of bacilli-form virus was clearly identified in the electron microscope but its RNA was not purified in the detectable amounts.

In fact, Harmsen *et al.* (1989) observed that a specific pattern of 10 major dsRNAs was always associated with diseased mushroom, *Agaricus bisporus* but non-diseased strains also contained at least one unique dsRNAs. Tavantzis *et al.* (1980), however, reported that a bacilli-form virus containing a ssRNA was extensively purified and characterized from unique symptomatic sporophores of a commercial mushroom, *A. bisporus*. They proposed that these MBV was the first unique mycovirus shown to possess a ssRNA genome. Compared to their finding of a high concentration of the 19×50-nm virus with relatively lower concentrations of two different spherical viruses, we could find two different sizes of bacilli-form viruses (16×47-nm and 4×23-nm) with higher concentrations of four different sizes of spherical viruses containing dsRNAs. In subsequent series of purification experiments, it was found that bacilli-form virus was still co-resolved from other spherical-form viruses at the differential equilibrium centrifugations in 15~35% performed sucrose density gradient and in a buyant density gradient of CsCl or Cs_2SO_4. We believe that at any case of virus preparation, the main banded components rep-

resented the spherical-form viruses and bacilli-form viruses since electron microscope examination of negatively stained virus preparations and their RNA patterns on agarose gel revealed their presence.

In case of healthy-looking crops of oyster mushrooms, they also contained dsRNAs with some variations. Laboratory strains of *P. ostreatus* (NFFA 2, NFFA 2 ml, NFFA 4001, and NFFA 4501) and *Pleurotus sajor-caju* strain did not contain any dsRNAs, whereas specific light scattering bands in the sucrose density gradient centrifugation were shown. However, mushrooms with specific disease symptoms contained at least four different sizes of spherical viruses and a single bacilliform virus. Thus, we could conclude that a bacilli-form virus may be a causative agent of abnormal growth.

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